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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number:	09/744,675
Applicants:	Edward L. Squires, Patrick M. McCue, George E. Seidel
Filed:	January 29, 2001
Title:	Equine System for Non-Surgical Artificial Insemination
TC/A.U:	1634
Examiner:	Carla J. Myers
Assignee:	XY, Inc.
Attorney Docket:	XY-Equine-USNP
Customer No.:	33549
Confirmation No.:	3456

APPEAL BRIEF PURSUANT TO 37 C.F.R. § 41.37

The Appellant files herein an Appeal Brief pursuant to 37 C.F.R. 41.37. A Final Office Action was issued in this Application on July 3, 2008. Pursuant to 37 C.F.R. 41.31, the Appellant filed a Notice of Appeal on December 30, 2008. Pursuant to 37 C.F.R. 41.37(a)(1), an Appeal Brief otherwise would have been due on February 28, 2009. The Appellant is requesting that this time period be extended for four months to and including June 30, 2009 pursuant to 37 C.F.R. 41.37(e). A Petition for Extension of Time along with the prescribed fee is filed together with this Appeal Brief. This Appeal Brief is filed under 37 C.F.R. 41.37 presently in effect as set forth in the Federal Register, Vol. 69, No. 155, pgs. 49960-50020, dated Thursday, August 12, 2004.

I. REAL PARTY IN INTEREST

The real party in interest in this appeal is XY, Inc., a wholly owned subsidiary of Inguran, LLC d/b/a Sexing Technologies, Inc.

II. RELATED APPEALS AND INTERFERENCES

Application No. 11/092,509, filed March 29, 2005 (Notice of Appeal Filed September 26, 2007). No decision has been rendered by the Board in this proceeding.

Application No. 09/879,480, filed June 12, 2001 (Notice of Appeal Filed April 28, 2009). No decision has been rendered by the Board in this proceeding.

III. STATUS OF CLAIMS

Canceled Claims: 1-137

Rejected/Objected Claims (Claims Now Under Appeal): 138-145

Claims Appealed: All claims other than those that have been canceled have been at least twice rejected; such claims are the ones subject to appeal at this time and include only claims 138-145. The Claims Appendix shows the claims in this proceeding as they stand at this time.

IV. STATUS OF AMENDMENTS

No amendments to the claims have been submitted subsequent to the Final Office Action dated July 3, 2008.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Generally, the inventive technology described in Appellant's independent claim 138 relates to the sorting of equine sperm cells based on sex characteristic. In natural service, it may be appreciated that a male equine generates a population of sperm cells having a roughly equal proportion of X-chromosome-bearing sperm cells to Y-chromosome-bearing sperm cells. The inventive technology discloses techniques for obtaining such a population of sperm cells from a male equine and sorting the population to obtain subpopulations having substantially all X-chromosome-bearing sperm cells or Y-chromosome-bearing sperm cells. Such subpopulations, having sperm cells of predominately one sex characteristic, then can be used to artificially inseminate a female equine to produce offspring having a preselected sex with a high degree of accuracy. Because equines are prize animals subject to intensive breeding programs, the ability to generate offspring having a preselected sex is highly useful.

The preamble of Appellant's independent claim 138 recites "establishing an equine artificial insemination sample." As discussed in Appellant's Application, an equine artificial insemination sample may require a relatively higher number of sperm cells for successful artificial insemination than those of other species. *See e.g.* Appellant's Application from page 2, line 26 to page 3, line 4. Additionally, as discussed in Appellant's Application, equine sperm cells often may be more delicate than those of other species. *See e.g.* Appellant's Application at page 5, lines 16-24.

Steps (a)-(j) of independent claim 138 involve sorting equine sperm cells by exploiting the fact that X-chromosome-bearing equine sperm cells have approximately 4.1 percent greater DNA content than Y-chromosome-bearing equine sperm cells. *See e.g.* Appellant's Application at page 13, lines 16-19. In certain embodiments, the technique of flow cytometery can be used to sort equine sperm cells based on this difference in DNA content. *See e.g.* Appellant's Application from page 13, line 20 to page 18, line 5; Figs. 1-2. Briefly, an unsorted population of equine sperm cells may be stained, such as with a fluorescent dye that binds to the DNA content of each equine sperm cell. The unsorted population of stained equine sperm cells may be introduced into a device called a flow cytometer. The flow

cytometer may act to entrain individual equine sperm cells on roughly a single-file basis into a moving flow of fluid called a sheath fluid. The flow cytometer further may have an oscillating nozzle through which the flow of sheath fluid may pass. The oscillating nozzle may cause the flow of sheath fluid to be broken up into individual droplets, each containing roughly one individual stained equine sperm cell. The droplets may then pass in front of the beam of a laser, which may cause the fluorescent dye bound to the DNA content of each equine sperm cell to fluoresce. Since the amount of dye taken up by an individual equine sperm cell is controlled by the DNA content of that equine sperm cell, X-chromosome-bearing equine sperm cells will fluoresce to a greater degree than Y-chromosome bearing sperm cells. The degree of fluorescence can be detected, for example by a photodetector, and X-chromosome-bearing equine sperm cells and Y-chromosome-bearing equine sperm cells can be sorted accordingly, for example by selectively electrically charging individual equine sperm cells and selectively electrically deflecting them into dedicated containers.

Step (g) of Appellant's independent claim 138 recites "separating… at a rate of at least nine hundred viable equine sperm cells per second", and step (i) of Appellant's independent claim 138 recites "collecting… at a rate of at least nine hundred viable equine sperm cells per second." These rates may be important when considered in light of the relatively high numbers of equine sperm cells perhaps necessary to maximize the effectiveness of artificial insemination in equines and the fact that sperm cells may be time-sensitive, perhaps tending to lose efficacy the longer they go unused. The ability to sort higher numbers of equine sperm cells in shorter periods of time therefore may be important. *See e.g.* Appellant's Application at page 2, lines 7-13; page 4, lines 12-18; page 10, lines 15-17; page 19, line 4 to page 21, line 3; page 36, line 29 to page 37, line 2.

Step (h) of Appellant's independent claim 138 recites "establishing a skim milk solution into which… said equine sperm cells entrained are collected." Such use of skim milk may exemplify an embodiment of the inventive technology wherein the collection fluid is used to mitigate aspects of the sorting process tending to induce stress in sperm cells. *See e.g.* Appellant's specification at page 21, lines 4-13. As discussed elsewhere in Appellant's Application, stress induced in sperm cells from the sorting process otherwise may tend to

adversely impact their performance when used for artificial insemination. *See e.g.* Appellant's Application at page 5, lines 23-27; page 19, line 24 to page 21, line 13.

Step (j) of Appellant's independent claim 138 recites "…fertilizing at least one egg *within* a female of said species of equine mammal" [emphasis added]. In this manner, the recitations of Appellant's claim 138 may be seen to recite *in vivo* fertilization. *See e.g.* Appellant's Application at page 6, lines 1-14.

Of course, the summary of the claimed subject matter described herein provides only a general overview. It is presented only to assist the reader in achieving a general conceptual understanding of the claimed subject matter and should not be construed to limit the disclosure of the Appellant's inventive technology.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following Grounds of Rejection are presented for review:

I. The Office's rejection of claims 138-140 and 142 under 35 U.S.C. 103(a) based on U.S. Patent No. 5,985,216 (hereinafter "Rens") in view of the non-patent literature by Wilhelm, *et al* (hereinafter "Wilhelm") and further in view of the non-patent literature by Rath, *et al* (hereinafter "Rath").

II. The Office's rejection of claim 141 under 35 U.S.C. 103(a) based on U.S. Patent No. 5,985,216 (hereinafter "Rens") in view of the non-patent literature by Wilhelm, *et al* (hereinafter "Wilhelm") and Rath, *et al* (hereinafter "Rath") and further in view of the non-patent literature by Catt, *et al* (hereinafter "Catt").

III. The Office's rejection of claim 143 under 35 U.S.C. 103(a) based on U.S. Patent No. 5,985,216 (hereinafter "Rens") in view of the non-patent literature by Wilhelm, *et al* (hereinafter "Wilhelm") and further in view of the non-patent literature by Rath, *et al* (hereinafter "Rath").

IV. The Office's rejection of claim 144 under 35 U.S.C. 103(a) based on U.S. Patent No. 5,985,216 (hereinafter "Rens") in view of the non-patent literature by Wilhelm, *et al* (hereinafter "Wilhelm") and further in view of the non-patent literature by Rath, *et al* (hereinafter "Rath").

V. The Office's rejection of claim 145 under 35 U.S.C. 103(a) based on U.S. Patent No. 5,985,216 (hereinafter "Rens") in view of the non-patent literature by Wilhelm, *et al* (hereinafter "Wilhelm") and further in view of the non-patent literature by Rath, *et al* (hereinafter "Rath").

VII. ARGUMENT

1. Claims 138-140 and 142

A. The combination of Rens, Wilhelm, and Rath do not teach the use of equine sperm cells as recited in Appellant's claims.

The Office concedes that "Rens does not specifically teach applying the sorting method to equine sperm," Office Action at page 4, but states "Wilhelm (page 321) teaches using equine sperm for artificial insemination methods and teaches methods for the effective cryopreservation of equine sperm," *Id.* However, "[t]o rely on a reference under 35 U.S.C. 103, it must be analogous prior art." MPEP § 2141.01(a). The Office is afforded a degree of latitude in determining what constitutes analogous art – "[u]nder the correct analysis, any need or problem known in the field of endeavor at the time of the invention and addressed by the patent [or application at issue] can provide a reason for combining the elements in the manner claimed." *Id., citing KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1397 (2007). Here, the Office cites Wilhelm as teaching the use of equine sperm for "artificial insemination." Office Action at page 4. However, a review of Wilhelm reveals that nowhere does Wilhelm use the equine sperm experimented on for artificial insemination. Rather, the purpose of Wilhelm is to determine if certain kinds of liposomes protect equine sperm from damage during cooling and freezing processes. *See e.g.* Wilhelm at pages 321, 322, and 326. Similarly, the Office cites Wilhelm as teaching "the effective cryopreservation of equine sperm." Office Action at page 4. This may be true, but the Appellant's claims do not recite any cryopreservation steps. Accordingly, other than the mere presence of equine sperm cells in Wilhelm, there is no overlap between the elements for which the Office cites Wilhelm ("artificial insemination," which Wilhelm does not teach, and "the effective cryopreservation of equine sperm," which Appellant does not claim) and the elements of Appellant's claims. As a result, Wilhelm is not analogous art and cannot be relied on by the Office to support a rejection under 35 U.S.C. § 103.

In addition, the Appellant notes that sorting equine sperm cells as recited in the Appellant's claims may pose different considerations than sorting bovine sperm cells as

taught in Rens, evidencing the lack of a "teaching, suggestion, or motivation" to combine Rens and Wilhelm as offered by the Office. *See generally* MPEP § 2143.01. Although the "teaching, suggestion, or motivation" test must not be applied in "an overly rigid and formalistic way," MPEP § 2141, *citing KSR International Co. v. Teleflex Inc.*, 82 USPQ2d 1385, 1391 (2007), nevertheless the Supreme Court has stated the test "capture[s] a helpful insight," *Id, citing KSR* at 1396, and that "[t]here is no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis," *Id.* Here, the issues associated with sorting equine sperm cells that are not present for sorting bovine sperm give rise to the lack of a "teaching, suggestion, or motivation" to combine Rens and Wilhelm as asserted by the Office. These issues are identified in Appellant's Application, and include the high numbers of equine sperm cells required for effective artificial insemination and the delicate nature of equine sperm cells as compared to other species. With respect to the high numbers of equine sperm cells required for effective artificial insemination, for example, Appellant's Application states:

> As mentioned, one of the fundamental challenges that efforts at sorting X and Y equine sperm has faced is the large numbers of sperm involved. In natural insemination equine sperm are produced by nearly the billion; in artificial insemination less, but still significantly large numbers of equine sperm are usually used. For instance, equine artificial insemination techniques routinely use two hundred and fifty million to five hundred million sperm. Thus a significant number of sperm have been presumed necessary in an equine artificial insemination environment.

See Appellant's Application from page 2, line 26 to page 3, line 4. With respect to the delicate nature of equine sperm cells, for example, Appellant's Application states:

> In the context of flow cytometry in general, most sorted cells or particles have often been physically able to withstand a variety of abuses. This is not the case for equine sperm cells. In fact, as the present invention discloses, the processing through normal flow cytometer techniques may, in fact, be unacceptable for cytometric sorting of equine sperm cells in certain applications. The sensitivities range from dilution problems and the flow cytometer's inherent need to isolate and distinguish each cell individually as well as the pressure and other stresses which typical flow cytometry has (prior to the present invention) imposed upon the equine cells it was sorting.

See Appellant's Application at page 5, lines 16-23. Because Wilhelm does not address these considerations in its use of equine sperm cells, and since Rens deals only with sorting bovine sperm cells, the lack of any other "teaching, suggestion, or motivation" to combine these references would further disincentivize a person of ordinary skill from doing so in the manner asserted by the Office, even above and beyond the fact that Wilhelm is not analogous prior art, as discussed above.

B. The combination of Rens, Wilhelm, and Rath do not teach separating viable equine sperm cells at a rate of at least 900 viable equine sperm cells per second.

The Office states that Rens teaches "sampling rates of 500 sperm/second and 2000 sperm/second (column 6)" and "sample rates up to at least 15,000 sperm/sec (column 4, lines 29-31)." Office Action at page 4. Naturally, "[t]he examiner bears the initial burden of factually supporting any *prima facie* conclusion of obviousness." MPEP § 2142. "Obviousness requires a suggestion of all limitations in a claim." *CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) (*citing In re Royka*, 490 F.2d 981, 985 (CCPA 1974); *see also In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (stating 35 U.S.C. § 103 requires "a searching comparison of the claimed invention – including all of its limitations – with the teachings of the prior art" and "entitles an applicant to issuance of an otherwise proper patent unless the PTO establishes that the invention *as claimed* in the application is obvious over cited prior art"). Because the *sampling rates* of Rens (*e.g.*, "sampling rates of 500 sperm/second and 2000 sperm/second (column 6)" and "sample rates up to at least 15,000 sperm/sec (column 4, lines 29-31)") are not the same as the *separation rates* recited in Appellant's claims (*e.g.*, "separating said droplets based upon said sex characteristic of said equine sperm cells entrained at a rate of at least nine hundred viable equine sperm cells per second" in Appellant's claim 138 (g)), Rens does not teach this element of Appellant's claims.

The distinction between *sampling rates* as described in Rens and the *separation rates* recited in Appellant's claims is evident from Rens itself. Rens states in relevant part that

"[s]perm preparation and staining were based on the method described by Johnson, *et al* [*Biol. Repro.* 41:199-203 (1989)]" (hereinafter "Johnson 1989"). Rens at column 4, lines 61-63. Johnson 1989's discussion of sample rates includes the following:

> Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15-20% of intact sperm pass through the laser beam with proper orientation (Fig. 1). In this study, the overall flow rate was approximately 2500 intact sperm/s. The intact X- and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80-90 X-bearing sperm/s and 80-90 Y-bearing sperm/s (Fig. 2).

Johnson 1989 at page 202. This passage from Johnson 1989 clearly illustrates that when the sample rate was 2500 intact sperm per second, the separation rate was only 80-90 X-bearing sperm per second and 80-90 Y-bearing sperm per second. Clearly, the separation rate is much lower than the sample rate.[1] Accordingly, it may be seen that simply knowing the sample rates of Rens, as cited by the Office, provides no information regarding the actual separation rate achieved by Rens. Rens therefore cannot teach the separation rates recited in the Appellant's claims (*e.g.*, "separating said droplets based upon said sex characteristic of said equine sperm cells entrained at a rate of at least nine hundred viable equine sperm cells per second" in Appellant's claim 138 (g)).

While the foregoing should serve to establish Appellant's contentions on this point, the following examples have been offered during prosecution and are presented again here to further illustrate that simple knowledge of the sample rates from Rens provides no information regarding the separation rates actually achieved by Rens:

> *Example 1 (from Appellant's Response and Request for Reconsideration dated November 1, 2006 in reply to the Office Action dated May 1, 2006):*

[1] For the curious reader, the cited passage from Johnson 1989 makes clear that in this case the separation rate is lower than the sample rate because no hydrodynamic orientation of the sperm cells is attempted, meaning that only 15-20 percent of the sperm cells passing before the flow cytometer's laser are in a proper orientation to accurately determine their sex characteristic. Johnson 1989 at page 202. It may be worth noting that hydrodynamic orientation is just one factor than can contribute to making the separation rate lower than the sample rate. Many other factors can contribute to this phenomenon as well, such as the coincidence rates discussed elsewhere herein.

The Office maintains an obviousness concern with respect to several combinations of references including Rens. However, Rens does not support the teachings for which the Office cites it. Specifically, the Office asserts that Rens' teaching of orientation rates of up to 60% and sampling rates of up to 2000 sperm per sec and 15,000 sperm per sec permit the conclusion that Rens' sorting rate is 1200 sorts per second (for sampling rates of 2000 sperm per sec) or 9000 sorts per sec (for sampling rates of 15,000 sperm per sec) [*see* Office Action dated May 1, 2006 at page 11]. It appears the Office's methodology for reaching this conclusion is as follows:

(sampling rate per second) x (percent of sperm oriented) = (sorting rate per second)

However, this methodology is not accurate in predicting the actual sorting rate per second. In particular, the sorting rate is dependent on other factors besides percent orientation and sampling rate. These factors tend to further compromise the sorting rate and result in sorting rates that are lower than those predicted by the Office's methodology. One example of such a factor is the phenomenon of coincidence rates. Coincidence occurs when more than one cell is entrained in a single droplet, resulting in an inability of a flow cytometer to make an accurate measurement of the droplet and thus the discarding of the cells without their being sorted. Moreover, factors such as coincidence rates tend to compound as the sample rate is increased, resulting in greater complications at sample rates of *e.g.* 15,000 sperm per second than for *e.g.* 2000 sperm per second. As a result, in the absence of specific data regarding the number of sorts per second achieved by Rens, it simply cannot be inferred what Rens' true sorting rate was.

This point is evidenced by the passages of Rens cited by the Office itself. Specifically, the Office cites Rens at column 4, lines 17-18 for teaching that the elliptical nozzle of Rens is capable of sorting in excess of 60% of sperm for sorting. The Office also cites Rens at column 4, lines 29-31 for teaching that proper orientation is maintained at sample rates up to at least 15,000 sperm per second. However, the same discussion in Rens at column 4, lines 24-27 notes that the elliptical nozzle of Rens achieves only a two-fold increase in efficiency as compared to a standard conical nozzle in combination with a beveled injection needle. By way of comparison, the use of such a beveled injection needle is described in US 5,135,759 to Johnson, *see e.g.* column 3, lines 27-41 and claim 12 [hereinafter "Johnson '759"]. When Johnson ['759] used the beveled injection tip to sort intact sperm, the sorting rate was only 80-90 sperm of each type per second at a sample rate of 2500 per second, as described in Johnson ['759] at column 4, lines 14-18. Therefore, since Rens notes that its elliptical nozzle achieves only a two-fold increase in efficiency as compared to a

standard conical nozzle with a beveled injection tip, it can be inferred that Rens' actual sorting rate was only on the order of 160-180 sperm per second for a sample rate of 2500 sperm per sec. This is far less than what would be predicted by the Office's methodology (2500 sperm per sec x 60% orientation = 1500 sorts per second), and is well below the sorting rates cited by the Appellant's claims.

Example 2 (from Appellant's Response and Request for Reconsideration dated November 1, 2006 in reply to the Office Action dated May 1, 2006):

A more detailed comparison can be made by reviewing Example 1 of Rens and comparing it to Johnson ['759]. First, note that Johnson ['759] states that approximately 15-20% of sperm are oriented when the beveled injection needle is used at a sample rate of 2500 sperm per second, *see* column 4, lines 10-15. Accordingly, using the Office's methodology, the predicted sorting rate would be 375-500 sorts per second:

(2500 sperm per sec) x (15% orientation) = 375 sorts per second

OR

(2500 sperm per sec) x (20% orientation) = 500 sorts per second

However, this prediction is incorrect. The data of Johnson ['759] clearly illustrate that the actual sorting rate achieved was only 80-90 sperm per second of each type, *see* column 4, lines 16-18. The discrepancy between the results predicted by the Office's methodology and the actual results is off by a factor of 4-6.

Next, note Rens at Example 1 teaches that at a sample rate of 2000 sperm per sec, the elliptical nozzle orients 52.5% of sperm. However, the question remains as to what the actual sort rate achieved was, as Rens provides no data on this point. Using the Office's methodology, the predicted sort rate for Rens should be 1050 sperm per second:

(2000 sperm per sec) x (52.5% orientation) = 1050 sorts per second

However, Example 1 of Rens used a sample rate of 2000 sperm per sec. In order to compare Rens' data to the sample rate of 2500 sperm per sec achieved by Johnson ['759], it is necessary to calculate what the sorting rate of Rens would be at a sample rate of 2500 sperm per second. Note that making this adjustment to Rens' sample rate should not introduce error, since Rens itself asserts at column 4, lines 29-31 that proper orientation is maintained at sample rates of up to at least 15,000 sperm per second. Using the Office's methodology, the new predicted sorting rate is 1312 sorts per second:

(2500 sperm per sec) x (52.5% orientation) = 1312 sorts per second

As stated previously, Rens provides no data on the actual number of sorts per second achieved. However, note that Rens in Example 1 does state that the orientation achieved with the elliptical nozzle (52.5%) is 2.3 times larger than the orientation achieved with the beveled injection tip (22.7%). Since data on actual sorts per second for a beveled injection tip at comparable sample rates is known from Johnson ['759], *see* Johnson ['759] at column 4, lines 14-18, it may be inferred that Rens' actual sorting rate is 2.3 times Johnson's ['759] actual sorting rate, or 184-207 sorts per second:

(Johnson: 80 sorts per second) x (Rens: 2.3 x improvement) = Rens: 184 sorts per second

OR

(Johnson: 90 sorts per second) x (Rens: 2.3 x improvement) = Rens: 207 sorts per second

So, the discrepancy between the sorting rate predicted by the Office's methodology (1312 sorts per second) and the inferred actual sorting rate achieved by Rens (at best, 207 sorts per second) is off by a factor of 6-7. Moreover, the inferred sorting rate of Rens again is well below the sorting rates recited by the Applicant's claims.

Importantly, note that the above calculations are not presented in an attempt to establish Rens' actual separation rate. As discussed, Rens' true separation rate cannot be known without explicit data from Rens that establishes that fact. Rather, the above calculations are presented merely to illustrate the point that the true separation rate of Rens cannot be determined simply from knowing Rens' sample rates, as the Office attempts to do. Because Rens does not disclose achieving the Appellant's claimed separation rates, it does not teach the element of the Appellant's claims for which it is cited. Since the Office has not factually shown that Rens achieved the Appellant's claimed separation rates, it has not carried its *prima facie* burden and it cannot use Rens to support the rejection under 35 U.S.C. § 103 that it seeks.

The Office states that "Rens (col. 7) also exemplifies a method wherein a total of 50 million X and Y bovine sperm were sorted in a 7 hour period…" Office Acton at page 4. While it is true Rens does present this information, nevertheless Rens at column 7 still does

not set forth any data regarding actual separation rates achieved. Following from the above discussion, the true separation rates achieved by Rens cannot be inferred from the passage cited by the Office. Moreover, it should be noted that Rens teaches only the sorting of bovine sperm. As discussed in this Appeal Brief at section VII(1)(A), the sorting of equine sperm cells may present considerations different than bovine sperm cells, such as the significantly larger number of sperm cells required for successful artificial insemination of equines and the more delicate nature of equine sperm cells as compared to bovine sperm cells. Consequently, in the absence of data from Rens specifically addressing separation rates for equine sperm cells, there is no way to know if the true separation rates achieved by Rens – whatever they may be – would be the same for equine sperm cells. Of course, the Office points out that Rens "states that the sorting method can be used with any mammalian sperm (column 4, lines 38-42)." Office Action at page 4. While this may be true, Appellant points out this statement of Rens only asserts that the technique can be applied to different mammalian sperm, and does not provide any information regarding what the separation rate for any given mammalian sperm would be.

C. Separating viable equine sperm cells at a rate of at least 900 viable equine sperm cells per second is not merely an optimization of a workable range.

The Office cites MPEP § 2144.05(b), "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation" and "discovery of an optimum value of a result effective variable in a known process is ordinarily within the skill of the art," as supporting its contention that "[i]t would have been obvious to one of ordinary skill in the art… to sort equine sperm at very high sort rates, including sorting rates that result in the collection of 900 viable sperm/second…" Office Action at page 5. While these provisions of the MPEP certainly are valid for the principles they assert, they are not applicable here because the recitations of Appellant's claims (*e.g.*, "separating said droplets based upon said sex characteristic of said equine sperm cells entrained at a rate of at least nine hundred viable equine sperm cells per second" in Appellant's claim 138 (g)) are not a case of optimizing values for known ranges.

First, for the reasons discussed in this Appeal Brief at section VII(1)(B), Appellant notes its claimed range of "at least nine hundred viable equine sperm cells per second" is not encompassed within nor overlaps any ranges of any combination of the references cited by the Office because all references disclose separating viable equine sperm cells only at less than 900 per second, if at all. "In the case where the claimed ranges '*overlap or lie inside ranges* disclosed by the prior art' a *prima facie* case of obviousness exists" [emphasis added]. MPEP § 2144.05. In fact, no separation rates disclosed in the references cited by the Office even approach Appellant's claimed range of "at least nine hundred viable equine sperm cells per second," and therefore cannot be considered even "close enough that one skilled in the art would have expected them to have the same properties." *See id.* Accordingly, Appellant's claimed range cannot be merely an optimization of known prior art conditions because the Office has not established a *prima facie* case that Appellant's claimed ranges overlap or lie within the ranges taught by the references.

Second, separating equine sperm cells at greater than 900 per second while maintaining viability of the sperm cells may pose considerations not present for separating sperm cells at lower rates. As stated in the Appellant's Application:

> One aspect of high speed sorting which appears to come into play when sorting equine sperm cells through a flow cytometer separation technique is that of the pressures and other stresses to which the equine sperm cells are subjected within the flow cytometer. For instance, when operating at high speeds (and an alternative definition of "high speed"), flow cytometers can be operated at a pressure of 50 pounds per square inch and even higher. These pressures may be considered high because they may result in effects upon the equine sperm cells being sorted… The key in this regard is that the stress imposed upon the equine sperm cells can, in fact, alter their viability and their ability to achieve the desired result. This may be unusually true for equine species. In the pressure case, it may be that merely subjecting the sperm cells to a higher pressure as a result of the operation of the flow cytometer at that pressure may result in decreased performance of the equine sperm cells. The present invention in one regard acts to minimize these stresses and thus results in greater efficacies as well as lower dosages as discussed later.

Appellant's Application from page 19, line 24 to page 20, line 11. These considerations identified in the Appellant's Application are not addressed in any of the references cited by

the Office, and therefore cannot be considered merely as "general conditions of a claim [that] are disclosed in the prior art." See MPEP § 2144.05(b). Rather, separating equine sperm cells at a rate of at least 900 per second while maintaining viability would require from a person of ordinary skill in the art more inventive effort than, for example, the mere routine adjustment of the flow rate of a flow cytometer – it would require the specific counteraction of the detrimental effects of high speed sorting on equine sperm cells. Compounding this task, of course, is the delicate nature of equine sperm cells, as discussed more fully in this Appeal Brief at section VII(1)(A). Accordingly, Appellant's claimed rate for separating equine sperm cells while maintaining their viability is not merely the "discovery of an optimum value of a result effective variable in a known process." *See* MPEP § 2144.05(b).

D. The combination of Rens, Wilhelm, and Rath does not teach viability as recited in the Appellant's claims.

The Office states that in Rens "the sperm is stained with Hoechst 33342 dye in order to distinguish between viable and nonviable sperm (column 5, lines 4-10)." Office Action at page 3. This is not true. Rens at column 5, lines 4-10 states that staining in this manner "allowed dead sperm to be distinguished from living sperm…" Rens at column 5, lines 8-10. While dead sperm certainly are *not* viable, not all live sperm *are* viable – there are instances where a sperm cell may be live but not viable. For example, live sperm cells may have broken tails, but may not be considered viable since the ability of a sperm cell without a functioning tail to fertilize an egg is compromised. *See also* Johnson 1989 at page 202 ("[i]ntact sperm (with tails), however, *whether viable or nonviable…*" – illustrating that even sperm with tails may be nonviable). The distinction between viable and nonviable equine sperm cells is more than merely academic, since fertilization success rates in artificial insemination procedures are directly linked to the number of viable equine sperm cells used. Since the portion of Rens cited by the Office does not teach the viable equine sperm cells for which the Office cites it, the combination of references does not teach viable equine sperm cells as recited in the Appellant's claims. Naturally, "obviousness requires a suggestion of all limitations in a claim." *CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) (*citing In re Royka*, 490 F.2d 981, 985 (CCPA 1974); *see also In re Ochiai*, 71 F.3d

1565, 1572 (Fed. Cir. 1995) (stating 35 U.S.C. § 103 requires "a searching comparison of the claimed invention – including all of its limitations – with the teachings of the prior art" and "entitles an applicant to issuance of an otherwise proper patent unless the PTO establishes that the invention as claimed in the application is obvious over cited prior art").

E. The combination of Rens, Wilhelm, and Rath do not teach the use of skim milk as recited in the Appellant's claims.

The Office states that "Rath teaches the concept of collecting sperm sorted cells into a sperm extender medium" and that "Wilhelm teaches extending equine sperm in skim milk solution…" Office Action at page 6. The Office's reasoning necessarily presumes that the skim milk solution of Wilhelm functions equivalently to the sperm extender medium of Rath. However, "[i]n order to rely on equivalence as a rationale supporting an obviousness rejection, the equivalency must be recognized in the prior art, and cannot be based on applicant's disclosure or the mere fact that the components at issue are functional or mechanical equivalents." MPEP § 2144.06, *citing In re Ruff*, 256 F.2d 590, 118 USPQ 340 (CCPA 1958). Here, there is no recognition of the required equivalency in the combination of references as asserted by the Office.

First, the teaching of Wilhelm applies to an extender for sperm cells prior to freezing, *see e.g.* Wilhelm at page 322, whereas the teaching of Rath (and the recitations of Appellant's claims) apply to a solution for collecting sperm cells after sorting, *see e.g.* Rath at page 796. Second, Wilhelm teaches use of a skim milk solution only as a medium for freezing and thawing equine sperm cells. *See e.g.* Wilhelm at page 322. Wilhelm even specifically describes its skim milk solution as a "freezing extender." *Id.* By way of contrast, the sperm cell extender of Rath is used only to collect sex sorted sperm. *See e.g.* Rath at page 796. Third, the skim milk solution in Appellant's Application is used to minimize stresses upon cells and provide for their easier collection after sorting, a purpose which nowhere is described in either Wilhelm or Rath. *See* Appellant's Application at page 21, lines 4-13. The Appellant's Application even states that "though intended for a different purpose, this extender can be used as the collection fluid for equine sperm cells." *See* Appellant's Application at page 21, lines 11-13.

These distinctions are significant. They point out differences in Wilhelm and Rath – the timing in which the extenders are used, and the differences in the collection and freezing applications for which the extenders are used, respectively – and neither Wilhelm nor Rath suggests that its composition would be suitable for the purposes described in the other. Further, neither Wilhelm nor Rath suggest that their compositions would be suitable for the purpose of minimizing stress to sorted equine sperm cells, as discussed in the Appellant's Application. Accordingly, the equivalency of the skim milk solution used in Wilhelm for the collection purposes disclosed in Rath is not supported by the references, and the Office has not met the burden for finding obviousness based on equivalency as set forth in MPEP § 2144.06.

F. The combination of Rens, Wilhelm, and Rath do not teach *in vivo* fertilization.

Appellant's claim 138 (j) recites the step of "…fertilizing at least one egg *within* a female of said species of equine mammal…" [emphasis added]. Naturally, "[t]he examiner bears the initial burden of factually supporting any *prima facie* conclusion of obviousness." MPEP § 2142. "Obviousness requires a suggestion of all limitations in a claim." *CFMT, Inc. v. Yieldup Intern. Corp.*, 349 F.3d 1333, 1342 (Fed. Cir. 2003) (*citing In re Royka*, 490 F.2d 981, 985 (CCPA 1974); *see also In re Ochiai*, 71 F.3d 1565, 1572 (Fed. Cir. 1995) (stating 35 U.S.C. § 103 requires "a searching comparison of the claimed invention – including all of its limitations – with the teachings of the prior art" and "entitles an applicant to issuance of an otherwise proper patent unless the PTO establishes that the invention as claimed in the application is obvious over cited prior art"). Here, each of the references cited by the Office teaches only *in vitro* fertilization, or at best does not provide enough information to establish that fertilization was *in vivo*. This is a significant difference, as *in vitro* fertilization generally is considered in the art to be more conducive for fertilization than *in vivo* fertilization. Accordingly, the combination of references does not teach the *in vivo* element of Appellant's claims and cannot be used to support a rejection under 35 U.S.C. § 103.

2. Claim 141

A. The combination of Rens, Wilhelm, Rath, and Catt do not teach a sheath fluid containing HEPES buffered medium as recited in Appellant's claims.

The Office concedes that Rens, Wilhelm and Rath "do not teach establishing a sheath fluid which contains a HEPES buffered medium," Office Action at page 9, but states "Catt teaches that semen may be diluted in a HEPES-buffered SOF (synthetic oviduct fluid) medium" and that "Wilhelm (page 321) does teach the use of a HEPES-buffered medium for extending equine sperm," *id.* The Office's reasoning necessarily presumes that the HEPES media of Catt and Wilhelm would function equivalently to the sheath fluid of Rens. However, "[i]n order to rely on equivalence as a rationale supporting an obviousness rejection, the equivalency must be recognized in the prior art, and cannot be based on applicant's disclosure or the mere fact that the components at issue are functional or mechanical equivalents." MPEP § 2144.06, *citing In re Ruff*, 256 F.2d 590, 118 USPQ 340 (CCPA 1958).

Here, there is no recognition of the required equivalency in the combination of references as asserted by the Office. As conceded by the Office, Rens, Wilhelm, and Rath do not teach a sheath fluid which contains a HEPES buffered medium at all. Moreover, the teachings of Catt and Wilhelm regarding HEPES media relate only to diluting and extending sperm cells, and do not teach the suitability of HEPES media for a sheath fluid. Accordingly, the equivalency of the HEPES media disclosed in Catt and Wilhelm for the sheath fluid disclosed in Rath is not supported by the references asserted by the Office in the manner required under MPEP § 2144.06.

In addition, the Appellant notes that Catt teaches sorting only ram and boar sperm, and Wilhelm does not teach sorting any sperm at all. Consequently, neither Catt nor Wilhelm address the considerations related to sorting equine sperm, discussed more fully in this Appeal Brief at section VII(1)(A). As a result, not only do Catt and Wilhelm fail to disclose the equivalence of their HEPES media to the sheath fluid of Rens, but they also fail to recognize the advantage of a HEPES buffered medium used as a sheath fluid for sorting equine sperm cells. This is a significant point. With respect to the references cited, only the Appellant's Application recognizes such an advantage. As stated in Appellant's Application:

> For the sheath fluid, a substance is selected according to one embodiment of the invention so that it may be chemically coordinated to present minimal changes. Thus, by selecting the appropriate sheath fluid not only in context of flow cytometry parameters, but rather also in context of the equine sperm cell and equine artificial insemination parameters themselves, the changes experienced by the cells and the over all result of the sorting can be enhanced. Interestingly, for equine sperm cells, it has been discovered that a hepes buffered medium such as a hepes bovine gamete medium — particularly HBGM3 as previously created by J. J. Parrish for a bovine application — works well. This medium is discussed in the article "Capacitation of Bovine Sperm by Heparin", 38 Biology of Reproduction 1171 (1988) (hereby incorporated by reference). Not only is this surprising because it is not the same type of use as for bovine sperm, but the actual buffer, was originally developed for a bovine application.

See Appellant's Application from page 20, line 20 to page 21, line 3. Accordingly, the combination of references cited by the Office not only fails to teach the actual use or equivalency thereto of a HEPES-buffered medium as a sheath fluid for sorting equine sperm cells in a flow cytometer, but also entirely ignores the reason why it is beneficial to do so, as is set forth in the Appellant's Application.

3. Claim 143

A. Operating a flow cytometer at 50 psi to sort viable equine sperm cells is not merely an optimization of a workable range.

The Office cites MPEP § 2144.05(b), "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation," as supporting its contention that "[t]o determine the optimum conditions for performing the sorting of sperm, including the sorting rates and pressure of the cell sorter is considered to be well within the skill of the art." Office Action at page 7. However, operating a flow cytometer at 50 psi to sort equine sperm cells while maintaining their viability is not merely the optimization of a workable range. This is because high pressure of this kind creates complicating factors that must be overcome in order to preserve viability of the equine sperm cells, which factors cannot be considered to be merely "general

conditions of a claim… disclosed in the prior art." *See* MPEP § 2144.05(b). As discussed in this Appeal Brief at section VII(1)(A), equine sperm cells may have a delicate nature as compared to other species. Additionally, Appellant's Application further notes the complicating effects of high pressures on equine sperm cells:

> One aspect of high speed sorting which appears to come into play when sorting equine sperm cells through a flow cytometer separation technique is that of the pressures and other stresses to which the equine sperm cells are subjected within the flow cytometer. For instance, when operating at high speeds (and an alternative definition of "high speed"), flow cytometers can be operated at a pressure of 50 pounds per square inch and even higher. These pressures may be considered high because they may result in effects upon the equine sperm cells being sorted… The key in this regard is that the stress imposed upon the equine sperm cells can, in fact, alter their viability and their ability to achieve the desired result. This may be unusually true for equine species. In the pressure case, it may be that merely subjecting the sperm cells to a higher pressure as a result of the operation of the flow cytometer at that pressure may result in decreased performance of the equine sperm cells. The present invention in one regard acts to minimize these stresses and thus results in greater efficacies as well as lower dosages as discussed later.

See Appellant's Application from page 19, line 24 to page 20, line 11. In this manner, it may be appreciated that operating a flow cytometer at 50 psi while preserving the viability of sorted equine sperm cells is not merely a case of determining an optimum range. Rather, a person having skill in the art would have to account and compensate for the increased stresses that are produced by such operation. Accordingly, the fact that the present invention allows a flow cytometer to be operated at 50 psi and still establish viable separated equine sperm cells capable of successful fertilization is not merely optimizing a workable range for known conditions.

4. Claim 144

A. An equine artificial insemination sample of no more than about 5 million equine sperm cells or no more than about 25 million equine sperm cells is not merely the selection of an optimum quantity of sperm.

The Office states that "it would have been obvious to one of ordinary skill in the art and well within the skill in the art to have selected an optimum quantity of sperm, wherein said quantity would be less than 25 million," because "Rens teaches that 4 to 5 million sorted sperm were used to inseminate dairy cows," "Rath (page 796 [*sic* – page 797] teaches the use of 0.2 million sorted porcine spermatozoa per oviduct," and "Rath teaches that approximately 3.5 to 4 x 10^5 sperm cells were sorted into each tube." Office Action at page 8. However, the requirement of MPEP § 2144.05(b) is that "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." Here, Rens and Rath do not disclose the general conditions applicable to Appellant's claimed recitations of equine artificial insemination samples of "no more than about five million equine sperm cells" and "no more than about twenty-five million equine sperm cells," and therefore these claimed values cannot be merely the selection of an optimum quantity sperm.

First, Appellant's claim of course recites only "equine sperm cells." By way of contrast, the combination of Rens and Rath teach only the use of bovine sperm cells and porcine sperm cells, respectively. As discussed in this Appeal Brief at section VII(1)(A), the sorting of equine sperm cells may present considerations different than bovine sperm cells and porcine sperm cells, such as the significantly larger number of sperm cells required for successful artificial insemination of equines and the more delicate nature of equine sperm cells. Accordingly, Rens and Rath do not teach as general conditions these aspects of equine sperm cells identified in the Appellant's Application, and therefore the methodologies of Rens and Rath cannot be relied upon to select optimum quantities of sperm in the equine context as recited in Appellant's claim.

Second, the methodologies themselves of Rens and Rath are substantially different from the disclosure of Appellant's Application. For example, as discussed more fully in this Appeal Brief at section VII(1)(F), both Rens and Rath utilize only *in vitro* fertilization or else do not provide sufficient information to identify the type of fertilization used. Moreover, specifically with respect to Rath, the sperm numbers cited by the Office either are not relevant to Appellant's claim or utilize a different technique than the Appellant. In particular, the "approximately 3.5 to 4 x 10^5 sperm cells" cited by the Office, Office Action at page 8, are

not relevant because they refer only to the numbers of sperm collected into tubes after sorting, Rath page 796, and are not the number of sperm cells in the "equine artificial insemination sample" recited by Appellant's claim. Similarly, the "0.2 million sorted porcine spermatozoa per oviduct" cited by the Office, Office Action at page 8, are used for surgical insemination, which differs from Appellant's Application. *See* Rath pages 796-797 (discussing slaughter of gilts, removal of reproductive tracts, puncturing of mature follicles with an 18-guage needle, *in vitro* fertilization, transfer of *in vitro* fertilized embryos, surgical exposure of the reproductive tracts of gilts, and surgical insemination of the *in vitro* fertilized embryos). This is quite different from the non-surgical artificial insemination techniques disclosed in Appellant's Application – pointedly, Appellant's techniques do not require the slaughter equines, the exposure of reproductive tracts, *etc*. *See e.g.* Appellant's Application at page 1, lines 6-8. Accordingly, the general conditions taught by Rens and Rath are substantially different than those disclosed in Appellant's Application, and therefore the methodologies of Rens and Rath cannot be relied upon to select optimum quantities of sperm in the equine context as recited in Appellant's claim.

5. Claim 145

A. An equine artificial insemination sample having a volume of 0.2 ml or 1 ml is not merely the selection of an optimum volume for sperm.

The Office states that "it would have been obvious to one of ordinary skill in the art and well within the skill in the art… to have selected the optimum volume for the artificial insemination sample," because Rath "teaches resuspending sorted porcine sperm in a solution having a volume of 0.2 ml." Office Action at page 8. However, the requirement of MPEP § 2144.05(b) is that "[w]here the general conditions of a claim are disclosed in the prior art, it is not inventive to discover the optimum or workable ranges by routine experimentation." Here, Rath does not disclose the general conditions applicable to Appellant's claimed recitations of an equine artificial insemination sample having "a volume selected from the group consisting of 0.2 ml, and 1 ml," and therefore these claimed values cannot be merely the selection of an optimum volume for an equine insemination sample.

First, Appellant's claim of course recites only "equine sperm cells." As discussed above at section VII(4)(A), Rath teaches only the use of porcine sperm cells. As discussed in this Appeal Brief at section VII(1)(A), the sorting of equine sperm cells may present considerations different than porcine sperm cells, such as the significantly larger number of sperm cells required for successful artificial insemination of equines and the more delicate nature of equine sperm cells. Accordingly, Rath does not teach as general conditions these aspects of equine sperm cells identified in the Appellant's Application, and therefore the methodology of Rath cannot be relied upon to select optimum volumes for equine artificial insemination samples as recited in Appellant's claim.

Second, the methodology itself of Rath is substantially different from the disclosure of Appellant's Application. For example, as discussed more fully in this Appeal Brief at section VII(1)(F), Rath utilizes only *in vitro* fertilization. Moreover, Rath's teaching of "resuspending sorted porcine sperm in a solution having a volume of 0.2 ml" is irrelevant to Appellant's claim. Appellant's claim recites "an equine artificial insemination sample," whereas the sperm of Rath resuspended to 0.2 ml is not utilized for artificial insemination. Rather, Rath teaches different values of sperm when discussing actual insemination. *See* Rath at page 797 ("4 x 10^5 sorted sperm cells/ml were added to the culture dish," discussing *in vitro* fertilization, and "100 ul TEST-yolk extender containing ~200,000 spermatozoa were placed into each oviduct," discussing surgical insemination). Of course, as discussed above at section VII(4)(A), the *in vitro* fertilization and surgical insemination techniques of Rath further create a set of general conditions substantially different from the *in vivo* fertilization and non-surgical insemination techniques disclosed in the Appellant's Application. Accordingly, the general conditions taught by Rath are substantially different than those disclosed in Appellant's Application, and therefore the methodologies of Rath cannot be relied upon to select the volume of sperm for an equine artificial insemination sample as recited in Appellant's claim.

VIII. CLAIMS APPENDIX

A Claims Appendix is attached immediately following, starting on the next page.

Filed via EFS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number:	09/744,675
Applicants:	Edward L. Squires, Patrick M. McCue, George E. Seidel
Filed:	January 29, 2001
Title:	Equine System for Non-Surgical Artificial Insemination
TC/A.U:	1634
Examiner:	Carla J. Myers
Assignee:	XY, Inc.
Attorney Docket:	XY-Equine-USNP
Customer No.:	33549
Confirmation No.:	3456

CLAIMS APPENDIX

138 A method of establishing an equine artificial insemination sample for equine artificial insemination comprising the steps of:

a. obtaining equine sperm cells from a male of a species of equine mammal;

b. staining said equine sperm cells to allow differentiation based upon a sex characteristic;

c. establishing a cell source which introduces said equine sperm cells which have been stained into a sheath fluid;

d. forming droplets in said sheath fluid;

e. entraining said equine sperm cells which have been stained in said droplets;

f. differentiating between said equine sperm cells entrained in said droplets based upon said sex characteristic;

g. separating said droplets based upon said sex characteristic of said equine sperm cells entrained at a rate of at least nine hundred viable equine sperm cells per second;

h. establishing a skim milk solution into which said droplets separated based upon said sex characteristic of said equine sperm cells entrained are collected;

i. collecting viable equine sperm cells separated based upon said sex characteristic in said skim milk solution at a rate of at least nine hundred viable equine sperm cells per second;

j. establishing an equine artificial insemination sample containing said viable equine sperm cells separated based upon said sex characteristic which are capable of fertilizing at least one egg within a female of said species of equine mammal.

139 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 138 wherein said step of establishing a skim milk solution into which said equine sperm cells are collected comprises the step of establishing a solution containing a skim milk extender as a collection fluid.

140 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 139 wherein said step of establishing a skim milk solution into which said equine sperm cells are collected further comprises the step of establishing a solution containing about four percent egg yolk as a collection fluid.

141 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 138 wherein said sheath fluid contains a HEPES buffered medium.

142 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 138 wherein said step of separating said droplets based upon said sex characteristic of said equine sperm cells further comprises the step of sorting said droplets having said equine sperm cells entrained using a flow cytometer.

143 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 142 wherein said step of sorting said droplets

having said equine sperm cells entrained comprises the step of operating said flow cytometer at a pressure of at least about fifty pounds per square inch.

144 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 138 wherein said equine artificial insemination sample is selected from the group consisting of: an equine artificial insemination sample of no more than about five million equine sperm cells, and an equine artificial insemination sample of no more than about twenty-five million equine sperm cells.

145 A method of establishing an equine artificial insemination sample for equine artificial insemination as described in claim 138 wherein said equine artificial insemination sample has a volume selected from the group consisting of : 0.2 ml, and 1 ml.

IX. EVIDENCE APPENDIX

An Evidence Appendix is attached immediately following, starting on the next page.

MPEP § 1205.02 states, "[i]f in his or her brief, appellant relies on some reference, he or she is expected to provide the Board with a copy of it in the evidence appendix of the brief." Accordingly, the Appellant includes copies of the following references in the Evidence Appendix:

U.S. Patent No. 5,985,216 to Rens (cited by the Office in the Final Office Action dated July 3, 2008).

Non-patent literature to Wilhelm, *et al* (cited by the Office in the Final Office Action dated July 3, 2008).

Non-patent literature to Rath, *et al* (cited by the Office in the Final Office Action dated July 3, 2008).

Non-patent literature to Catt, *et al* (cited by the Office in the Final Office Action dated July 3, 2008).

Non-patent literature to Johnson, *et al* (discussed in U.S. Patent No. 5,985,216 to Rens, cited by the Office in the Final Office Action dated July 3, 2008).

U.S. Patent No. 5,135,759 to Johnson (cited by the Appellant in its Response and Request for Reconsideration dated November 1, 2006 in reply to the Office Action dated May 1, 2006).

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number:	09/744,675
Applicants:	Edward L. Squires, Patrick M. McCue, George E. Seidel
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Examiner:	Carla J. Myers
Assignee:	XY, Inc.
Attorney Docket:	XY-Equine-USNP
Customer No.:	33549
Confirmation No.:	3456

EVIDENCE APPENDIX

US005985216A

United States Patent [19]

Rens et al.

[11] Patent Number: 5,985,216

[45] Date of Patent: Nov. 16, 1999

[54] **FLOW CYTOMETRY NOZZLE FOR HIGH EFFICIENCY CELL SORTING**

[75] Inventors: **Willem Rens**, Norfolk, United Kingdom; **Glenn R. Welch**; **Lawrence A. Johnson**, both of Silver Spring, Md.

[73] Assignee: **The United States of America, as represented by the Secretary of Agriculture**, D.C., Wash.

[21] Appl. No.: **08/898,999**

[22] Filed: **Jul. 24, 1997**

[51] **Int. Cl.**[6] **G01N 33/48**

[52] **U.S. Cl.** **422/73**; 422/81; 422/99; 422/100; 356/23; 356/246; 356/336; 239/423

[58] **Field of Search** 422/73, 81, 99, 422/100; 356/23, 246, 336, 337; 239/423, 424

[56] **References Cited**

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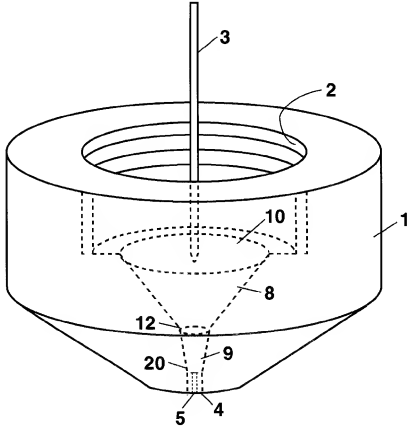
Primary Examiner—Arlen Soderquist

Attorney, Agent, or Firm—M. Howard Silverstein; Curtis P. Ribando; John D. Fado

[57] **ABSTRACT**

A sorting nozzle having two tapered zones with elliptical cross-sections is able to orient and sort a large fraction of asymmetrical or "flattened" cells. This nozzle has particular application for sorting viable male (Y) and female (X) sperm populations in a cell sorter.

7 Claims, 3 Drawing Sheets



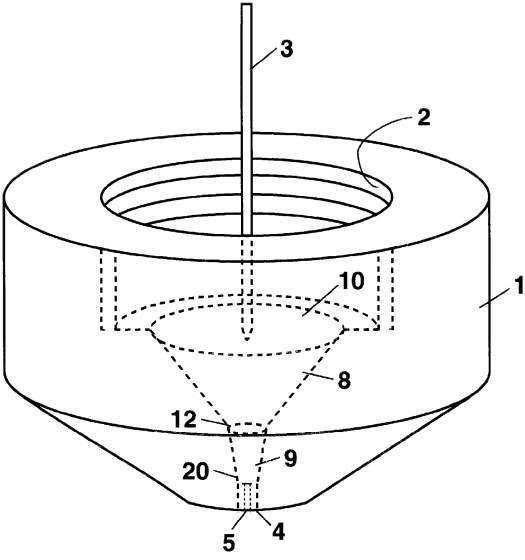


Fig. 1

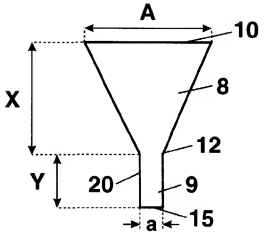


Fig. 2A

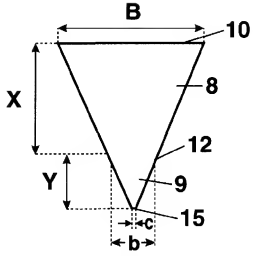


Fig. 3A

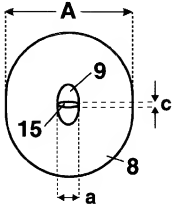


Fig. 2B

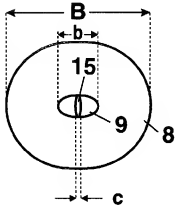


Fig. 3B

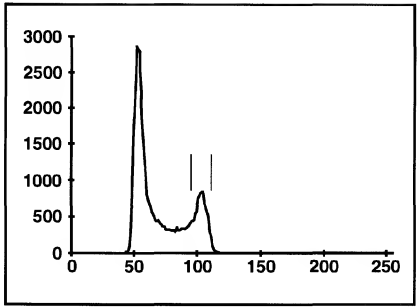

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Fig. 4A

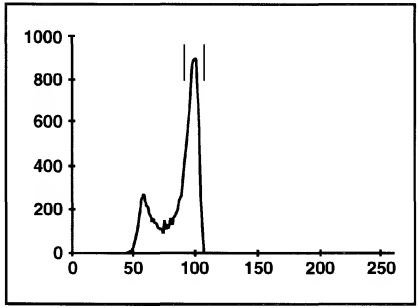
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Fig. 4B

FLOW CYTOMETRY NOZZLE FOR HIGH EFFICIENCY CELL SORTING

BACKGROUND OF THE INVENTION

1. Field of the Invention

The physical separation of semen into purified X- and Y-chromosome bearing sperm populations using flow cytometry and sorting has proven to be effective in humans, cattle, swine, sheep and rabbits for gender preselection.

The success of the sorting process is dependent on the accuracy and efficiency of analyzing sperm for DNA content. High resolution flow cytometric DNA analysis of sperm is hampered by its uneven emission of fluorescence. Due to the sperm head's flat shape, compactness of chromatin and a high index of refraction, fluorescence from the edge is much brighter than from the flat side of the sperm (Gledhill et al., *J Cell Physiol.* 87:367–376 (1976); Johnson et al., *Cytometry* 7:268–273 (1986); and Pinkel et al., *Cytometry* 3:1–9 (1982)]. This fact, coupled with random orientation, results in a broad fluorescence distribution hiding specific subpopulations with different contents of DNA within a sample. A solution for this problem is to use an epiilluminated flow cytometer employing co-axial flow characteristics. However, this system is generally not suitable for cell sorting. The only solution that incorporates DNA analysis and sorting is to collect only fluorescence from properly oriented cells using an orthogonal flow cytometer/cell sorter (Johnson et al. and Pinkel et al., supra). In the orthogonal system, a 90° fluorescence detector is used for detection of properly oriented sperm. A bright signal indicates that a sperm's bright edge is effectively orientated to the 90° detector and consequently the flat side will face the laser beam. A forward fluorescence detector (0°) is added to the cell sorter to collect the fluorescence from the flat face opposite to the laser beam. The forward fluorescence signal when collected from the flat side of the properly orientated sperm is an accurate measurement of DNA content (Johnson et al. and Pinkel et al., supra).

Improved high efficiency sorting can only be valuable for sperm sexing if three criteria are met:

1) It does not decrease the accuracy of the DNA measurement (maintain 90% purities).
2) Stability of sort is not compromised (time not wasted adjusting cell sorter).
3) Enough sperm can be recovered enabling their use for in vitro fertilization and artificial insemination.

This invention relates to a novel nozzle which is designed to orient and sort a large fraction of sperm independent of sheath fluid velocity and sample rate.

2. Description of the Prior Art

The orientation of sperm (cells) is random in conventional cell sorters. For sperm sexing, cell sorters have been modified by replacing the sample injection needle with a beveled needle [Dean et al., *Biophys. J.* 23:7–13 (1978); Fulwyer, *J. Histochem. Cytochem.* 25:781–783 (1977); Johnson et al., supra; Pinkel et al., supra; Stovel, et al., *Biophys. J.* 23:1–5 (1978); and Welch et al., *Cytometry* 17(Suppl. 7): 74 (1994)]. This beveled needle forces a larger proportion of sperm to pass the laser beam in the proper orientation because it reshapes the cylindrical sample stream into a thin ribbon. The beveled needle helps to orient sperm, especially sperm heads, e.g., sperm without their tail. The sample core leaving the beveled needle will be in the shape of a ribbon, which applies orienting forces to the sperm. However this ribbon only exists when the sample stream is narrow; that is, under low sample pressure and concomitant low sample rate. These conditions are not advantageous for efficient sperm sorting. Also, the improvement in orientation attributed to use of a beveled needle is less pronounced for living and motile sperm. Only 20–40% of intact viable sperm are correctly orientated using this system [Johnson, *Reprod. Fertil. Dev.* 7:893–903 (1995)]. This means that between 60% and 80% of the detected sperm are not analyzed for DNA analysis. The ability to sort intact and viable cells in conjunction with improved orientation efficiency would lead to a much higher sorting efficiency.

Kachel et al. [*J. Histochem. Cytochem.* 25:774–780 (1977)] disclose an asymmetric Plexiglas® chamber for orienting flat particles (fixed chicken erythrocytes) on a microscope. Kachel et al. suggest that the simplest flow path for applying the necessary hydrodynamic focusing forces consists of a tube with an elliptical cross-section and ending in an elliptical outlet having a long axis at right angles to the long axis in the cross-section of the constricting elliptical tube. This device was not proposed for use in combination with a cell sorter.

SUMMARY OF THE INVENTION

We have now discovered a novel nozzle design useful specifically for high efficiency flow sorting of asymmetrical or "flattened" cells in a cell sorter. The nozzle's unique design incorporates two ellipsoidal interior zones and an elliptical exit orifice capable of stable droplet formation and cell sorting. A unique performance characteristic of the nozzle of the invention is that, when used with intact viable sperm, the proportion of oriented sperm are essentially independent of sperm motility, sample rate, and sheath stream velocity.

In accordance with this discovery, it is an object of the invention to provide a cell sorter nozzle that will improve the efficiency of correctly orienting nonradially symmetrical cells in a separation stream.

It is a specific object of the invention to provide a means for improving the accuracy and efficiency of sperm cell sorting in a flow cytometer.

It is another object of the invention to enable the utilization of high speed cell sorters in order to maximize the number of sorted sperm per unit time.

Other objects and advantages of the invention will become readily apparent from the ensuing description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a perspective view of one embodiment of the nozzle of the invention.

FIG. **2**A is a front elevation interior outline of the nozzle of the invention.

FIG. **2**B is a top view of the interior outline of the nozzle as depicted in FIG. **2**A.

FIG. **3**A is a side elevation interior outline of the nozzle of the invention.

FIG. **3**B is a top view of the interior outline of the nozzle as depicted in FIG. **3**A.

FIG. **4**A is an orientation histogram of boar intact sperm measured with a beveled needle/conical nozzle system of the prior art.

FIG. **4**B is an orientation histogram of boar intact sperm measured with a conventional cylindrical needle/elliptical nozzle in accordance with the invention.

DETAILED DESCRIPTION OF THE INVENTION

The novel nozzle design of the invention is most typically embodied as a stream jet-in-air nozzle which is described in

the ensuing discussion. However, it would be appreciated by a person of ordinary skill in the art that the design could be incorporated into other types of nozzles as well, such as a quartz channel nozzle.

Referring to FIG. **1**, the nozzle essentially comprises a body **1** having interior threads **2** or other suitable means for attachment to the flow cell body or nozzle holder (not shown). In an alternate embodiment, a pressure fitting could be used instead of mating threads for attaching the nozzle to the holder. The interior of the nozzle is adapted to receive sample injection needle **3**, which is adjusted to extend into the nozzle a sufficient depth to maintain laminar flow. The injection needle may be a standard cylindrical needle or it may be beveled. In the tip of the nozzle, opposite the means for attachment, is a cylindrical orifice **5** which serves as the final exit from the flow nozzle. Orifice **5** is typically drilled into a jewel **4** which is positioned at the end of nozzle body **1**.

Referring to FIGS. **2A**, **2B**, **3A** and **3B**, the interior of the nozzle comprises a first ellipsoidal zone **8** and a second ellipsoidal zone **9** separated by a transition zone **12**. Virtually all cross-sections of the first and second zones and of the transition zone **12** are each in the shape of an ellipse having a long axis and a short axis. The first zone **8** is tapered from the mouth to the transition zone such that the cross-sectional area of said transition zone is less than that of the mouth. The second zone is tapered from the transition zone to an exit orifice **15** having a cross-sectional area less than that of the transition zone. The long axis of the exit orifice ellipse is oriented approximately 90° to the long axes of both the mouth and the transition zone. Orifice **5** is immediately downstream from the elliptical exit orifice **15**. Orifice **5** is concentrically aligned with exit orifice **15** and also has a smaller cross-sectional area than the exit orifice. The interior shape of nozzle **1** can be visualized with a standard flexible funnel by compressing opposite sides of the bowl of the funnel to form an oval shape at the mouth, and by compressing the stem of the funnel at 90° to the compressive forces applied to the bowl to form an elliptical slit at the stem outlet.

The actual and relative dimensions of the nozzle interior in a preferred embodiment of the invention are given in Table I, below. The dimension references are found in FIGS. **2A**, **2B**, **3A**, and **3B**. Dimensions A and p represent the short and the long axes, respectively, of mouth **10**. Dimensions a and b represent the short and the long axes, respectively, of transition zone **12**. Dimension a also represents the long axis of exit orifice **15**. Dimension c represents the short axis of the orifice. Dimension X is the height of first zone **8** and Y is the height of second zone **9**. The dimensions given in Table I are considered to be approximately optimal for the purpose of orienting viable intact sperm. However, it would be appreciated by the person in the art, that these dimensions could be varied within reasonable limits without significantly altering the functional ability of the nozzle to sort while orienting viable sperm or other asymmetrical cells or objects. The tolerances for these dimensions are at least about ∓10% of the values given in Table I.

Table II presents ratios of various significant nozzle dimensions based upon the relationships given in Table I. These ratios may vary within limits of ∓20%, with the proviso that the ratio of B/A remains greater than 1 so that mouth **10** is elliptical. Typically, the ratio of B/A should be at least about 1.1. In comparing the ratio B/A to the ratio b/a, it is apparent that the relative dimensions of the long axis to the short axis of cross-sections of the first zone progressively increase from the mouth of the zone to the transition zone. In succeeding cross-sections downstream from the transition zone, that is, in the second zone, the long axis/short axis ratio begins to decline. At an "inversion" point **20** which is intermediate transition zone **12** and exit orifice **15**, the axes are equal and the interior cross-section of the second zone is circular. From the inversion point to the exit orifice, cross-sections of the second zone are again elliptical; but the long axes of the cross-sections are at 90° to the long axes of the cross-sections above the inversion point. For the ellipses inscribed by each succeeding cross-section approaching the exit orifice, the ratios of the long axes to the short axes progressively increase. It is understood that in each progressively downstream cross-section from the mouth of the nozzle to the exit office, the dimensions of both the long axis and the short axis decrease commensurate with the continuous tapering of both the first and second zones.

The elliptical nozzle of this invention is capable of orienting in excess of 60% of sperm for sorting. When compared to a conventional system employing a standard cylindrical nozzle in combination with a standard conical sample injection needle wherein sperm traverse the laser beam with random orientation, the elliptical nozzle achieves approximately a three-fold increase in the proportion of sperm oriented for sorting. Approximately a two-fold increase in efficiency is obtained in comparison with a standard conical nozzle in combination with a beveled injection needle. When a beveled needle is used to orient sperm, the fraction of oriented sperm decreases with increasing sample rate. We have found that with the elliptical nozzle, the proportion of proper orientation is maintained at sample rates up to at least 15,000 sperm per second. This high level of performance is beneficial for efficient sperm sorting. In tests with bovine sperm (Example 5), it is apparent that a cell sorter equipped with the new nozzle effectively separates X- and Y-chromosome bearing sperm into sorting purities of approximately 90%.

The nozzle of the invention is envisioned to be useful for sorting viable sperm of any mammalian species as well as for sorting blood cells, seeds and other asymmetrical units. Tests with bovine sperm (Example 2) indicate that performance of the nozzle is not influenced by sperm motility.

A high speed sorter equipped with the nozzle of this invention increases the yield of sorted X- and Y-chromosome bearing sperm 10-fold and will make artificial insemination with sexed sperm a more feasible alternative to in vitro fertilization and embryo transfer or surgical insemination in gender preselection of livestock and other animals.

The following examples are intended to further illustrate the invention.

EXAMPLES

Sperm Preparation and Staining

Ejaculated semen from several mature bulls and boars of proven fertility and on regular collection schedules, and semen from mature New Zealand White bucks (rabbit) were used for this study. In addition, one semen sample each of the mouse and human was evaluated for demonstration purposes because of the differing head shapes of their sperm. Sperm preparation and staining were based on the method described by Johnson et al. [*Biol. Repro.* 41:199–203 (1989)] and Johnson et al. [*Gamete Research* 17:203–212 (1987)]. Briefly, aliquots of neat semen were extended to a concentration of $15–10^9$/ml to $100×10^9$/ml in: Hepes buffered medium containing 0.1% BSA (pH=7.4) for bull sperm, Beltsville Thawing Solution (BTS, pH=7.2) for boar sperm,

and Tris buffer (0.21 M Tris, 58 mM glucose, and 67 mM citric acid; pH=6.9) for rabbit sperm. Mouse and human semen were extended in BTS. Sperm were subsequently stained with 7.1 μM Hoechst 33342 per 15×10^6 sperm (Calbiochem-Behring Corp., La Jolla, Calif.) and incubated over a 40-min period at 32° C. For the bull sperm studies, just prior to analysis propidium iodide (PI) (1.5 μM, Calbiochem-Behring Corp., La Jolla, Calif.) was added to the Hoechst 33342 stained sperm. This allowed dead sperm to be distinguished from living sperm as described by Johnson et al. (1994, supra).

Cell Sorting

For Examples 1–5, two variations of a standard cell sorting system were used for sorting viable intact sperm with the nozzle of invention: the EPICS® V series flow cytometer/cell sorter (Coulter Corporation, Miami Fla.) and the EPICS® 750 (a modified version of the EPICS® V). For Examples 6 and 7, a newer system, the MoFlo® high speed cell sorter (Cytomation Inc., Fort Collins, Colo.) was used. Each cell sorter was modified for sorting sperm as described in Johnson et al. (1986), supra. The primary modification is the replacement of the forward light scatter diode detector with a forward fluorescence detector, necessary for orienting and sorting intact and viable sperm. The modification allows the collection of fluorescence from the brighter edge of the sperm (existing 90° fluorescent detector, which selects the proportion of sperm orientated properly) as well as the dimmer face of the sperm whose fluorescence is collected by the forward detector only from orientated sperm. The standard EPICS® uses a 76 μm conical flow nozzle which was replaced by the elliptical nozzle of the invention having the measurements indicated in Table I. The MoFlo® high speed system was adapted with a complete flow cell system common to the EPICS® system and was then able to receive the elliptical nozzle. Sperm intersected the laser beam after passing through the stream jet-in-air nozzle. The nozzle alone is responsible for orienting the sperm and can do so with either the standard cylindrical needle or with a beveled needle. The fluorochrome of the stained sperm was excited with ultraviolet light.

To fully demonstrate the efficacy of this invention, several experiments were conducted. The first 5 experiments (Examples 1–5) outlined below were designed to quantify (Student's t-test for small samples) the improvement in orientation and sorting obtained by the nozzle of the invention. The last two experiments (Examples 6 & 7) applied the nozzle of the invention to the high efficiency sorting of X and Y sperm for fertilization which would otherwise not have been possible.

When purities of the sorted samples are reported, the percentages were determined by the flow cytometric reanalysis of the sorted sample. Aliquots (100,000 to 250,000 sperm) of sperm were pulse sonicated to remove tails (allowing for better precision in analysis). Sperm were then restrained and rerun on the flow cytometer. Resulting histograms were fit with a double Gausian curve fitting routine.

Example 1

In this Example, the performance of the nozzle of the invention was compared with a standard sperm sorting system and with a system using a beveled needle with an inclusive angle of 12° at the outlet end. Semen of 8 different bulls was analyzed on two different days with the standard conical nozzle and standard cylindrical injection needle. Semen of 15 different bulls was analyzed on three different days and measured with: 1) the standard conical nozzle and beveled needle, 2) the elliptical nozzle and standard cylindrical needle.

Sample rates were about 2000 sperm per second. Window settings to select oriented sperm signals were the same for each experiment. Measurements of sperm with the standard and elliptical nozzles were done with two different cell sorters (EPICS® V and EPICS® 750). In this way, low proportions of oriented sperm caused by changing needles backwards and forwards was avoided.

A large improvement in bull sperm orientation was achieved using the elliptical nozzle compared with the standard nozzle fitted with a cylindrical or beveled needle (paired difference t-tests, $p<0.05$, separate data not shown). On average, a 3.0 times larger proportion of oriented sperm was obtained with the elliptical nozzle (52.5±4.7%, n=15) when compared with standard conical nozzle in combination with the standard cylindrical needle (17.3±0.7%, n=8) and a 2.3 times larger proportion of oriented sperm when compared with the standard nozzle in combination with the beveled needle (22.7±3.5%, n=15).

Example 2

This example was designed to analyze the influence of sperm motility on orientation by the elliptical nozzle of the invention. Motility is an important feature of viable sperm, as only motile sperm samples are considered suitable for sexing and fertilization. Proportions of oriented intact viable sperm were compared with proportions of oriented sperm without their tails and proportions of oriented dead intact sperm. Measurements were performed on four different days with semen of different bulls. Sample rates were about 2000 sperm per second. Viable sperm could be analyzed separately from dead sperm by their differential Hoechst fluorescence, because PI, which only stains dead sperm, quenches Hoechst fluorescence.

Proportions of oriented viable bull sperm (56.8±6.7%) were the same as those of tailless bull sperm (59.8±4.2%) or dead bull sperm (53.3±4.3%, no significant difference at $p=0.05$, n=4), showing that motility has a negligible influence on orientation of bull sperm when the novel elliptical nozzle is used.

Example 3

This example was designed to assess the influence of sample rate on orientation. Sample rate is important as it is advantageous to sort sperm in the shortest amount of time possible. Semen of 8 bulls was measured on two days with sample rates of 500 sperm/sec and 2000 sperm/sec. Orientations of 52.1±6.4% and 52.3% ±6.5 were obtained for sample rates of 500 sperm/sec. and 2000/sec. (n=8, paired difference t-test), respectively, demonstrating that orientation was not influenced by sample rate.

Example 4

In Example 4, sperm collected from rabbits, mice and humans were evaluated to investigate the orientation performance of the elliptical nozzle for these species with contrasting sperm morphology. Semen of 3 different rabbits was analyzed with the elliptical nozzle and proportions of correctly oriented sperm were determined. Additionally, mouse sperm and frozen-thawed human sperm were analyzed to demonstrate the use of this nozzle for these species.

A high proportion of rabbit sperm were correctly oriented when measured with the elliptical nozzle (48±2.4% n=3). The proportion of oriented sperm obtained with mouse sperm and human cryopreserved-thawed sperm was 44% and 45% respectively.

Example 5

This example was designed to test the ability of the novel nozzle system for producing sufficient numbers of sorted populations of sperm from two different species that could then be shipped by air to different regions of the country and used the same day for in vitro fertilization of oocytes at that particular location. High purity sorted samples were obtained on all days of the experiment (Table III). On average, a X-sperm sort purity of 87.6% ±3.1% and a Y-sperm sort purity of 89.3±2.5% were obtained for borine sperm.

Boar sperm orientation on average was characterized by a X-sperm sort purity of 85.3±2.10% and a Y-sperm sort purity of 89.4±2.4% was obtained (Table IV). Proportions of orientated sperm were on average 60.5±0.9%. Orientation histograms obtained with the conical nozzle/beveled needle system and with the elliptical nozzle/cylindrical needle are presented in FIGS. **4**A and 4B, respectively, to show the significant improvement in orientation. The paired vertical bars in the figures represent the orientation window required for effective sorting of X- and Y-chromosome bearing sperm.

Example 6

Example 6 demonstrates that sufficient sperm could be sorted in a short time, yet in sufficient magnitude to use the sorted sperm for regular artificial insemination in cattle. Two million sorted X and Y sperm using both boar and bull sperm needed to be sorted and recovered at approximately 90% purities (85–95%) between 9am and noon and 8am and 10am respectively. These constraints were imposed because of required counter to counter air shipment times which enabled same day in vitro fertilization (IVF) by Research collaborators in other states.

The sorts described above were successfully carried out 4 times for boar IVF and 6 times for bull IVF. The previous sorting technology which only allowed several hundred thousand sperm to be recovered per hour would have made these experiments impossible to do.

Example 7

Sorted X and Y (primarily X) sperm were used for artificial insemination of dairy cows. Four to five million sperm were required at approximately 90% purity (85–90%) for each recipient. Up to 5 recipients were to be artificially inseminated per day with sorted sperm. Therefore, up to 25 million×sperm (50 million, total X and Y) were required to be sorted between 9am and 4pm. This would not have been possible without the nozzle of the invention. These experiments (from 1 to 5 cows) were successfully carried out 5 times.

TABLE I

Parameters of Preferred Embodiment

	A	B	a	b	c	X	Y
Actual (mm)	6	7	0.9	2	0.2	5.5	2.5
Relative to c	30c	35c	4.5c	10c	—	27.5c	12.5c

TABLE II

Significant Dimensional Ratios of Preferred Embodiment

Relationship	Ratio	Minus 20%	Plus 20%
B/A	1.2	>1.0	1.4
b/a	2.2	1.8	2.6
a/c	4.5	3.6	5.4
X/A	0.9	0.7	1.1
Y/a	2.8	2.2	3.4
A/a	6.7	5.4	8
B/b	3.5	2.8	4.2

TABLE III

Sort purities of bovine (bull) X-sperm and Y-sperm measured with the elliptical nozzle

Day	Orientation (%)	X-sort (%)	Y-sort (%)
1	50	88.0	90.5
2	57	91.5	90.5
3	59	84.0	85.5
4	52	87.0	90.5
Mean ± sd	54.5 ± 4.2	87.6 ± 3.1	89.3 ± 2.5

TABLE IV

Sort purities of porcine (boar) X-sperm and Y-sperm measured with the elliptical nozzle

Day	Orientation	X-sort (%)	Y-sort (%)
1	61.5	84.0	89.0
2	60.0	84.3	87.3
3	60.0	87.7	92.0
Mean ± sd	60.5 ± 0.9	85.3 ± 2.1	89.4 ± 2.4

We claim:

1. A nozzle comprising a body having a first interior zone and a second interior zone, said first and second zones being separated by a transition zone, wherein essentially all cross-sections of said first and second zones and said transition zone are in the shape of an ellipse having a long axis and a short axis, wherein said first zone comprises a mouth and is tapered from said mouth to said transition zone such that the cross-sectional area of said transition zone is less than that of the mouth and the ratio of the long axis to the short axis increases as the cross-sectional area of the first zone decreases from the mouth to the transition zone, wherein said second zone is downwardly tapered from said transition zone to an exit orifice having a cross-sectional area less than that of the transition zone and the ratio of the lone axis to the short axis decreases from the transition zone to a point within the second zone where said lone and short axes are equal and thereafter the ratio of the long axis to short axis increases and the lone axis is at approximately 90° to the lone axis in both of said mouth and said transition zone, and wherein the long axis in said exit orifice is at approximately 90° to the long axes in both of said mouth and said transition zone.

2. The nozzle of claim **1** wherein said nozzle further comprises means for attachment to a flow cell.

3. The nozzle of claim **2** wherein said means for attachment are interior threads adjacent to said first zone and adapted to be received by exterior threads on said flow cell.

4. The nozzle of claim **2** wherein said means for attachment is a pressure fitting.

5. The nozzle of claim 1 wherein said body further comprises a cylindrical orifice downstream from, and of smaller cross-section than, said exit orifice.

6. The nozzle of claim 1 wherein dimensions A and B are the short and the long axes, respectively, of said mouth, dimensions a and b are the short and the long axes, respectively, in said transition zone, dimension a is also the long axis in said exit orifice, dimension c is the short axis in said exit orifice, dimension X is the height of said first zone and dimension Y is the height of said second zone and the ratio of B/A is in the range of about 1.1–1.4, the ratio of b/a is in the range of about 1.8–2.6, the ratio of a/c is in the range of about 3.6–5.4, the ratio of X/A is in the range of about 0.7–1.1, the ratio of Y/a is in the range of about 2.2–3.4, the ratio of A/a is in the range of about 5.4–8.0, and the ratio of B/b is in the range of about 2.8–4.2.

7. The nozzle of claim 1 wherein dimensions A and B are the short and the long axes, respectively, in said mouth, dimensions a and b are the short and the long axes, respectively, in said transition zone, dimension a is also the long axis in said exit orifice, dimension c is the short axis in said exit orifice, dimension X is the height of said first zone and dimension Y is the height of said second zone and the value of A is about 6 mm, the value of B is about 7 mm, the value of a is about 0.9 mm, the value of b is about 2 mm, the value of c is about 0.2 mm, the value of X is about 5.5 mm and the value of Y is about 2.5 mm.

* * * * *

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Effects of Phosphatidylserine and Cholesterol Liposomes on the Viability, Motility, and Acrosomal Integrity of Stallion Spermatozoa Prior to and after Cryopreservation

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Computer-assisted motion analyses (CASA) and flow cytometry were used to evaluate stallion spermatozoa prior to and after cryopreservation. Spermatozoa were pretreated with: (1) Hepes-buffered medium (SHB); (2) phosphatidylserine (PS) liposomes; or (3) liposomes composed of both PS and cholesterol (PSCH) prior to dilution in either SHB or skim milk–egg yolk extender (SMEY). After cooling to 5°C in SHB, PS and PSCH pretreatment resulted in higher percentages of motile spermatozoa (42 and 43%, respectively) than SHB pretreatment (23%). Spermatozoal motion parameters were higher for spermatozoa diluted in SMEY than dilution in SHB. In Experiment 2, motion parameters were compared for spermatozoa pretreated with PSCH liposomes and cryopreserved in either SMEY or a high salt-skim milk–egg yolk extender (CO). Spermatozoal motion characteristics were similar for all spermatozoal treatments after cooling to 5°C. After cryopreservation, PSCH liposome-treated samples had higher percentages of motile spermatozoa than untreated samples regardless of freezing extender. Samples frozen in CO medium had higher percentages of motile spermatozoa than samples frozen in SMEY ($P < 0.05$; 63% in CO + PSCH and 54% in CO vs 55% in SMEY + PSCH and 48% in SMEY, respectively). In Experiment 3, spermatozoa were treated with dilauroylphosphatidylcholine (PC12) to induce the acrosome reaction. The percentages of viable cells and viable acrosome-reacted spermatozoa were higher for fresh spermatozoa than for cryopreserved spermatozoa ($P < 0.05$), but were not affected by PSCH liposome treatment ($P > 0.05$). Addition of PSCH liposomes improved recovery of motile spermatozoa after cryopreservation but did not affect the ability of spermatozoa to undergo a PC12-induced acrosome reaction. © 1996 Academic Press, Inc

Cryopreservation can damage subcellular compartments of spermatozoa resulting in decreased fertilizing potential (17). Perturbations in membrane structure occurs during cooling to 5°C and during cryopreservation (27). This alters the ability of spermatozoa to maintain ion concentration gradients (29, 36) and maintain Na^+/K^+ ATPase activity (19), and can lead to potentially lethal increases in intracellular Ca^{2+} concentration (36). These changes may also adversely affect spermatozoal survival in the female tract (34).

Addition of exogenous lipids, such as egg yolk, prevent many of these physiological changes from occurring during the cooling and freezing processes (28). It has been determined that a phospholipid component of the low density lipoprotein fraction of egg yolk specifically protects spermatozoa during cooling to 5°C (21, 30, 35). Purified phosphatidylserine (PS) has also been shown to protect boar (2) and bull (11) spermatozoa from cold shock damage. In addition, liposomes composed of PS and cholesterol (PSCH) protected bull (11) and stallion (38) spermatozoa from cryopreservation damage, apparently by preventing some of the detrimental changes which occur to spermatozoa during the freezing and thawing process.

Flow cytometry has been used to analyze the viability (9, 10) and the acrosomal integrity (15, 26) of spermatozoa. This technique enables multiple spermatozoal parameters to be measured simultaneously on thousands of individual cells in only a few minutes (3). Recently, techniques have been developed to measure plasma membrane integrity and acro-

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somal integrity of bull spermatozoa simultaneously, using propidium iodide (PI), to evaluate plasma membrane integrity, in conjunction with a fluorescently labeled lectin, *Pisum sativum* agglutinin (PSA), to measure acrosomal membrane integrity (15, 26). PI is a nuclear stain which cannot pass through an intact plasma membrane, and spermatozoa possessing intact plasma membranes prohibit PI nuclear staining. The PSA lectin binds to both α-mannose and α-glucose residues of the acrosomal contents identifying spermatozoa possessing acrosomal membranes which are not intact (7).

The present study utilized both computer-assisted spermatozoal analysis (CASA) and flow cytometry to determine if liposomes composed of PS alone or PSCH increase the motility, viability, and acrosomal integrity of stallion spermatozoa prior to and after cryopreservation.

MATERIALS AND METHODS

Experiment 1

This experiment was conducted to evaluate the ability of PS and PSCH liposomes to protect stallion spermatozoa from cold shock and freezing damage.

Liposome preparation. Liposomes were made as previously described (11). Briefly phosphatidylserine (7 mg) alone or with an equal amount of cholesterol (7 mg each) dissolved in chloroform was placed into test tubes and the tubes were purged with nitrogen gas to remove the chloroform. A 12-ml volume of a sugar-based, Hepes-buffered medium (SHB: 277.5 m*M* glucose, 8.3 m*M* lactose, 5.0 m*M* raffinose, 1.7 m*M* sodium citrate dihydrate, 2.5 m*M* potassium citrate, 29.8 m*M* Hepes, pH 7.2) was added to the tubes and the liposomes were made by forcing this solution through 0.2-μm pores of an Extruder filter (Lipex Biomembranes, Inc., Vancouver, BC). Mayer *et al.* (23) reported that liposomes prepared using this technique were unilamellar vesicles having diameters of 179 $\pm$ 55 nm when vesicle size was determined by quasi-elastic light scatter techniques. The liposomes were stored in 1-ml aliquots at -80°C until needed. Liposomes were thawed in a 39°C water bath and used immediately. One batch of liposomes was used for the entire experiment. We have previously used many different batches of liposomes to freeze sperm (11), and all have consistently produced similar effects on sperm; a single batch was used in these studies to ensure that all replicates received identical concentrations of liposomes.

The concentration of vesicles in solution was not measured. However, since vesicle size was uniform measurement of liposome constituents should reflect liposome concentration. The concentration of phosphatidylserine in the PS and PSCH liposome solutions were determined (416 and 432 μM, respectively) using ammonium ferrothiocyanate (32). Cholesterol content was not measured, as sufficient cholesterol was added to completely saturate the PS molecules, resulting in a 1:2 molar ratio of cholesterol:PS (25).

Spermatozoal collection and preparation. Spermatozoa were collected from 18 stallions using an artificial vagina and immediately extended 1:1 (v/v) in SHB prewarmed to 37°C. Spermatozoa were placed into 15-ml conical centrifuge tubes and centrifuged at 300*g* for 8 min, after which the supernatant was removed by aspiration. Spermatozoal pellets were resuspended in 0.5 ml SHB and all the spermatozoa for an individual stallion were pooled. The spermatozoal concentration was determined by spectrophotometric assay (16) and the spermatozoa were diluted to 800 $\times$ 10^6 cells/ml in SHB, and subsequently divided into six aliquots each of 300 μl.

Two of these spermatozoal aliquots were each treated with 500 μl of: (1) SHB, (2) PS liposomes in SHB (final PS concentration of 260 μM), or (3) PSCH liposomes in SHB (final PS concentration of 260 μM) and gently agitated to assure complete mixing of the additive with the spermatozoa. Spermatozoa were incubated with liposomes for 15 min at 23°C. This incubation time allowed adequate interaction of liposomes with bull spermatozoa (11).

After incubation with the liposomes, 4 ml

SHB (containing 4% (v/v glycerol) or 4 ml SMEY (154.8 m*M* glucose, 4.2 m*M* lactose, 2.5 m*M* raffinose, 0.85 m*M* sodium citrate dihydrate, 1.25 m*M* potassium citrate, 29.8 m*M* Hepes, 2% egg yolk by volume, 4% (v/v) glycerol) was added to spermatozoa pretreated with SHB, PS, or PSCH (final spermatozoal concentration of 50×10^6 cells/ml). Spermatozoa were placed into a 100-ml water bath at 23°C and cooled to 5°C over 2.5 h at an initial cooling rate of −0.2°C/min. When the temperature of the samples reached 5°C, spermatozoa were packaged into 0.5-ml polyvinylchloride straws (IMV International. Minneapolis, MN) and placed in a freezing chamber (40 cm diameter × 35 cm deep) containing 6 liters of liquid nitrogen and the straws were frozen in static nitrogen vapor 7 cm above the liquid nitrogen for 15 min (initial freeze rate of −540°C/min). Straws were then plunged into liquid nitrogen and stored at −196°C. Spermatozoa were analyzed immediately after thawing in a 39°C water bath for 30 s.

Computer-aided spermatozoal motion analysis. The percentage of motile spermatozoa in each sample was determined after cooling to 5°C and again after cryopreservation, using a Stromberg-Mika motion analyzer (Bad Feilbach, Germany; settings were 30 frames/16 ms; depth of field = 16 μm; head size minimum area = 30 pixels, maximum area = 120 pixels; motile cells must travel >20 μm/s) equipped with a slide warmer maintained at 37°C and at least 200 spermatozoa per sample were analyzed. The percentage of motile spermatozoa and the straight-line velocity (VSL) and the average-path velocity (VAP) of motile sperm were determined.

Experiment 2

This experiment was conducted to evaluate the ability of PSCH liposomes to protect spermatozoa from cryopreservation damage when spermatozoa were frozen in extenders containing either high or low concentrations of salts.

Spermatozoal collection and preparation. Semen from 11 stallions was used to compare the motion parameters of spermatozoa treated with or without PSCH liposomes and then extended in two different skim milk–egg yolk extenders. The experimental design and the procedures used in this experiment were similar to those from Experiment 1 with the exception that a modified Tyrode's-based skim milk–egg yolk extender developed in Colorado (CO: 18.5 m*M* NaCl, 5 m*M* KCl, 0.6 m*M* KH_2PO_4, 17.85 m*M* $NaHCO_3$, 1.2 m*M* $MgSO_4$, 5 m*M* Hepes, 8.5 m*M* $CaCl_2{}^2H_2O$, 42 m*M* fructose, 18.55 m*M* glucose, 0.6 m*M* Na pyruvate, 6.55 m*M* Na lactate, 1.5 mg/ml BSA, 5.15 g/100 ml skim milk, 2% (v/v) egg yolk, 3% (v/v) glycerol) was used as a freezing extender as well as SMEY.

Spermatozoa were washed in SHB as previously described and resuspended to 800×10^6 cells/ml in SHB. Four 300-μl aliquots of spermatozoa were combined with 500 μl of either SHB or PSCH and the spermatozoa were gently agitated to assure complete mixing of the samples. After 15 min at 23°C, 4 ml CO or 4 ml SMEY was added to the spermatozoal samples (final spermatozoa concentration = 50×10^6 cells/ml), resulting in four spermatozoal treatments: (1) SMEY, (2) CO, (3) PSCH + SMEY, and (4) PSCH + CO. The spermatozoa were then cooled, frozen, and thawed, and motion parameters were assessed by CASA as described in Experiment 1.

Experiment 3

The previous two experiments indicated that PSCH liposomes protected stallion spermatozoa from cold shock and freezing damage. This experiment was conducted to determine if addition of liposomes would alter the ability of spermatozoa to undergo the acrosome reaction.

Spermatozoal collection and preparation. Spermatozoa from seven stallions were collected and washed as described in Experiment 1. Spermatozoa were resuspended to a concentration of 800×10^6 cells/ml and either PSCH liposomes or a modified Tyrode's medium (STALP: 37 m*M* NaCl, 10 m*M* KCl, 1.2 m*M* KH_2PO_4, 35.7 m*M* $NaHCO_3$, 2.4 m*M* $MgSO_4$

anhydrous, 10 m*M* Hepes, 17 m*M* $CaCl_2{\cdot}2H_2O$, 84 m*M* fructose, 5.5 m*M* glucose, 1.2 m*M* Na pyruvate, 13.1 m*M* Na lactate, 3 mg/ml BSA, pH 7.4) was added to the spermatozoa as described previously and the spermatozoa were incubated for 15 min at 23°C, diluted with 4 ml of CO medium, and frozen as described in Experiment 1.

Induction of the acrosome reaction. Fresh and frozen–thawed spermatozoa were prepared identically to induce the acrosome reaction and to analyze the spermatozoa by flow cytometry. Frozen–thawed spermatozoa from seven straws (3.5 ml) or 3.5 ml fresh spermatozoa was diluted with 8 ml STALP and centrifuged at 125*g* for 10 min. Spermatozoal pellets were resuspended in STALP to a concentration of 40×10^6 cells/ml. This washing procedure removed seminal plasma from fresh spermatozoa and egg yolk droplets from the frozen–thawed spermatozoa, which would interfere with the ability of dilauroylphosphatidylcholine (PC12) to induce the acrosome reaction. PC12 was added to spermatozoa samples at final concentrations of 0, 20, 40, 60, or 80 μM as described by Graham and Foote (11) and samples were incubated at 39°C for 15 min to induce the acrosome reaction.

Flow cytometric assay of the acrosome reaction. Spermatozoa were evaluated for vesiculation of the apical portion of the head indicative of the acrosome reaction (assessed by the binding of PSA lectin) and for permeabilization of the plasma membrane (assessed by DNA uptake of propidium iodide). Spermatozoa were stained with 1.2 μg/ml PI and 75 μg/ml phycoerythrin-conjugated PSA for 4 min prior to flow cytometric analysis.

The fluorescence intensity of each probe was determined for 50,000 cells, using an Epics V flow cytometer (Coulter Electronics, Hiahleah, FL) fitted with a beveled tip to orient the spermatozoa properly as they passed through the sensor (20). Fluorescence was excited at 488 nm by an argon laser (100 mW). Fluorescence was detected using a filter setup which included a 515-nm interference filter, a 560-nm dichroic beam splitter filter, a 560-nm shortpass filter to detect the PSA, and a 630-nm longpass filter to detect PI. Spermatozoa were separated into four different categories: (a) viable cells with intact acrosomes (PI−, PSA−); (b) viable cells with damaged acrosomes (PI−, PSA+); (c) nonviable cells with intact acrosomes (PI+, PSA−); (d) nonviable cells with damaged acrosomes (PI+, PSA+).

Statistical Analysis

Percentage data in all experiments were transformed by arcsine and all spermatozoal parameters were analyzed using analysis of variance (31). Treatment means were separated using Student–Newman–Keuls multiple range test, with a significance level of 0.05 (31).

RESULTS

Experiment 1

Spermatozoal motion parameters, after initial liposome treatment and dilution in either SHB or SMEY, after cooling to 5°C, and after cryopreservation, are presented (Table 1). Samples incubated with PS and PSCH liposomes and subsequently extended in SMEY medium exhibited higher percentages of motile spermatozoa initially (81 and 84%, respectively) than spermatozoa extended in SHB without liposome treatment (63%; $P < 0.05$). Treatment with liposomes also resulted in higher percentages of motile spermatozoa when spermatozoa were extended in SHB and cooled to 5°C (42 and 43%) than spermatozoa not treated with liposomes (23%; $P < 0.05$). Liposome treatment did not affect the percentage of motile spermatozoa when spermatozoa were extended in SMEY and cooled to 5°C (Table 1). After cooling, spermatozoa extended in SMEY treatments maintained higher percentages of motile spermatozoa (64–68%) than spermatozoa extended in SHB (23–43%).

Samples cryopreserved in SMEY medium exhibited higher percentages of motile spermatozoa than spermatozoa cryopreserved in SHB, despite treating the spermatozoa with liposomes (46–58% vs 12–17%; $P < 0.05$).

TABLE 1

Motion Parameters of Stallion Spermatozoa Processed in a Sugar, Hepes-Buffered Extender (SHB), and a Skim Milk-Egg Yolk Extender (SMEY); with or without the Addition of Phosphatidylserine (PS) or Phosphatidylserine + Cholesterol (PSCH) Liposomes, after Initial Dilution, after Cooling to 5°C, and after Cryopreservation

		Initial (N = 13)			Cooled (N = 21)			Frozen (N = 19)		
Extender	Treatment	Mot (%)	VSL (μm/s)	VAP (μm/s)	Mot (%)	VSL (μm/s)	VAP (μm/s)	Mot (%)	VSL (μm/s)	VAP (μm/s)
SMEY	PSCH	84 a	76 a	79 a	68 a	76 a	78 a	58 a	68 a	70 a
	PS	81 a	78 a	81 a	64 a	76 a	79 a	47 b	68 a	70 a
	None	77 a, b	77 a	80 a	67 a	76 a	78 a	46 b	72 a	74 a
SHB	PSCH	68 a, b	64 b	67 b	43 b	57 b	59 b	17 c	39 b	40 b
	PS	70 a, b	63 b	66 b	42 b	50 b	52 b	16 c	37 b	39 b
	None	63 b	64 b	67 b	23 c	41 b	42 b	12 c	19 c	19 c
SEM		4	2	2	3	5	5	3	5	5

Note a, b, and c denote differences within columns at $P < 0.05$. The percentage of motile spermatozoa (mot), the straight-line velocity (VSL), and average-path velocity (VAP) were characterized for the different treatments. (N, number of ejaculates per treatment.)

Although samples treated with PSCH liposomes and then cryopreserved in SMEY exhibited higher percentages of motile spermatozoa than spermatozoa subjected to other treatments, treatment of spermatozoa with PS liposome did not improve postthaw percentages of motile spermatozoa.

Spermatozoal velocity characteristics after initial dilution, after cooling, and after cryopreservation followed similar patterns as the percentages of motile spermatozoa at the three temperatures. Straight-line and average path velocities were higher for spermatozoa extended in SMEY after cooling and after cryopreservation than for spermatozoa extended in SHB medium (Table 1). Addition of liposomes did not affect VSL or VAP of the spermatozoa cryopreserved in SMEY medium, but resulted in higher postthaw spermatozoal velocities when spermatozoa were frozen in SHB medium ($P < 0.05$).

Experiment 2

The motion parameters of spermatozoal samples pretreated with PSCH liposomes and subsequently extended in either CO or SMEY medium after cooling to 5°C and after cryopreservation are presented in Table 2. Spermatozoal motion parameters were similar in all spermatozoal treatments after cooling to 5°C ($P < 0.05$). After freezing and thawing, however, spermatozoa pretreated with PSCH liposomes and cryopreserved in CO medium exhibited higher percentages of motile spermatozoa (63%) than spermatozoa cryopreserved in SMEY without liposome addition (48%). Within each extender, the addition of PSCH liposomes improved postthaw percentages of motile spermatozoa ($P < 0.05$). In addition, cryopreservation of spermatozoa in CO medium resulted in higher postthaw percentages of motile spermatozoa than the SMEY medium ($P < 0.05$). The average path velocities were similar for motile spermatozoa regardless of spermatozoa treatment ($P > 0.05$). However, after cryopreservation, spermatozoal VSL was highest for spermatozoa pretreated with PSCH liposomes and frozen in the CO medium (80 μm/s).

Experiment 3

The percentages of viable spermatozoa and viable acrosome-reacted spermatozoa after incubation with PC12, as determined by flow cytometric analysis, for spermatozoa extended in CO medium and for spermatozoa pretreated

TABLE 2

Motion Parameters of Stallion Spermatozoa Cooled to 5°C and Frozen in a Skim Milk–Egg Yolk Extender (SMEY) or a Tyrode's Based Skim Milk–Egg Yolk Extender (CO) after Addition of Phosphatidylserine + Cholesterol (PSCH) Liposomes ($N = 11$)

		Cooled			Frozen[a]		
Extender	Treatment	Mot (%)	VSL (μm/s)	VAP (μm/s)	Mot (%)	VSL (μm/s)	VAP (μm/s)
CO	PSCH	84 a	85 a	88 a	63 a*	80 a	83 a
	None	84 a	88a	92 a	54 a,b	76 a,b	79 a
	Mean	*84*	*87*	*90*	*59***	*78*	*81*
SMEY	PSCH	79 a	75 a	78 a	54 a, b*	70 b	73 a
	None	79 a	83 a	86 a	48 b	74 a, b	76 a
	Mean	*79*	*79*	*82*	*51*	*72*	*75*
SEM		4	4	4	3	3	3

Note. a, b, and c denote differences within columns ($P < 0.05$) excluding extender means in italics.

[a] Two replicates per stallion for frozen semen analyses.

* Denotes difference between extender with and without liposomes ($P < 0.05$)

** Denotes difference between extender means (mean in italics) in spermatozoal motion parameters ($P < 0.05$).

with PSCH liposomes and then extended in CO medium, after initial extension and after cryopreservation are presented (Fig. 1). Addition of PSCH liposomes did not affect the percentage of viable spermatozoa or the percentage of live acrosome-reacted spermatozoa either prior to or after cryopreservation ($P > 0.05$). Neither was viability of fresh or frozen–thawed spermatozoa affected by PC12 treatment. However, PC12 did induce acrosome reactions in both nontreated and PSCH-treated spermatozoa both prior to and following cryopreservation. In addition, the percentage of the live spermatozoa population exhibiting an acrosome reaction was similar across all extender treatments at each PC12 concentration (13–14, 15–32, 22–41, 39–45, and 53–60% of the live spermatozoa population exhibiting acrosome reactions when treated with 0, 20, 40, 60, and 80 μM PC12, respectively).

DISCUSSION

Since the initial report of Phillips and Lardy (28), egg yolk has been added to spermatozoal preparations to minimize cold shock and cryopreservation damage. Subsequent research determined that the phospholipids in the low density lipoprotein fraction of the egg yolk were specifically responsible for spermatozoal protection from cold shock damage (21, 30, 35). Although egg yolk lipoproteins appear to associate with spermatozoal membrane lipids and remain with the spermatozoa after repeated washings by centrifugation (8), washing renders the spermatozoa susceptible to cold shock damage (30). After much investigation, the mechanism by which egg yolk phospholipids interact with spermatozoal membranes remains unclear.

Liposomes composed of purified PS and PSCH prevent spermatozoal cold shock damage during cooling to 5°C (2, 11) and protect spermatozoa during cryopreservation (11, 38). The spontaneous transfer of PS to erythrocyte cell membranes (6) and fibroblast cell membranes (22) has been reported. The mechanism by which PS and PSCH liposomes interact with the spermatozoal membrane, however, remains unclear because neither PS nor PSCH liposomes adhere to or fuse with fresh bull spermatozoa (1, 33), and the mechanism by which these liposomes protect sperm may be as difficult to discern as that for egg yolk.

Some of the factors involved in cell survival

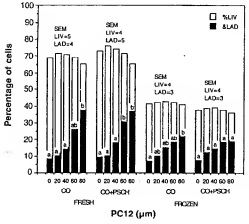

FIG. 1. Stallion spermatozoa treated with five different concentrations (0, 20, 40, 60, and 80 μM) of PC12 liposomes prior to and after cryopreservation. Spermatozoa had been previously incubated with or without PSCH liposomes. Parameters measured included the percentages of viable spermatozoa (LIV) and the percentages of viable acrosome reacted (LAD) spermatozoon. Differences between PC12 concentrations within extender treatment (CO or CO + PSCH) are denoted by a and/or b and are significant at $P < 0.05$.

during cryopreservation include maintaining a sufficient fraction of unfrozen water in which cells can reside as well as limiting medium hypertonicity (24). Spermatozoa are sensitive to hypertonicity, particularly if exposed to hypotonic conditions prior to freezing (37). It has been suggested that lipids may help prevent sperm cold shock by chelating calcium ions in the medium, preventing calcium entry into spermatozoa (36). It may be possible that liposomes interact with calcium and other components of the freezing medium that affects medium tonicity or the fraction of unfrozen water during cryopreservation. Our experiments shed no light on such interactions. However, the possibility of liposome interactions with lipid, protein, and ionic constituents of the medium needs to explored.

The purpose of this study was to determine if liposomes composed of PS and PSCH protect stallion spermatozoa from damage during cooling and freezing. Unlike previous studies using bull spermatozoa (11), treatment of stallion spermatozoa with PS and PSCH liposomes did not maintain percentages of motile stallion spermatozoa, after cooling to 5°C, to the same degree as the combination of egg yolk and skim milk. However, liposome treatment was beneficial to spermatozoa diluted in the SHB medium (containing no egg yolk or milk). Part of the reason that stallion spermatozoa treated with liposomes did not respond similarly to bull spermatozoa may be due to species differences in spermatozoal membrane composition, which may affect the capacity of liposomes to protect spermatozoa from cold shock damage. Part of this difference may also be due to the sire populations of these two species. Bulls, at artificial insemination centers, have been selected and screened for the ability of their spermatozoa to survive during the cryopreservation process, whereas the stallions used in this study had no such selection or screening.

Likewise, PS or PSCH liposomes by themselves failed to protect stallion spermatozoa from freezing damage. Again, this is different from previous studies using bull spermatozoa, where PSCH liposomes protected bull spermatozoa from freeze–thaw damage as well as egg yolk (11). However, PSCH liposomes in combination with egg yolk and skim milk together resulted in higher percentages of motile stallion spermatozoa after thawing than spermatozoa frozen in SMEY alone.

The different spermatozoal treatments affected spermatozoal motion characteristics after initial dilution, after cooling to 5°C, and after cryopreservation. Liposome addition to spermatozoa resulted in higher spermatozoal velocities after cryopreservation when spermatozoa were frozen in the SHB medium. However, liposome treatment did not affect spermatozoal velocity for spermatozoa frozen in SMEY medium. The egg yolk and skim milk may contain components which protect spermatozoa membranes and provide additional energy sources or nonsubstrate molecules that stimulate velocity of spermatozoal movement, masking the beneficial effects that liposomes have on the spermatozoa.

In Experiment 2, PSCH liposome treatment resulted in higher postthaw percentages of motile spermatozoa regardless of freezing extender used. This indicated that liposome interactions with stallion spermatozoa are independent of the sugar or salt composition of the extender. The composition of the freezing extender also influenced spermatozoal recovery after cryopreservation. The CO medium resulted in higher percentages of motile spermatozoa and higher spermatozoal velocities than the SMEY medium, indicating that higher salt concentrations in the cryopreservation medium may be beneficial to stallion spermatozoa.

Both skim milk–egg yolk-based extenders used in this study resulted in higher percentages of motile spermatozoa than has been reported for an EDTA–egg yolk extender (4, 5). However, differences in spermatozoal processing techniques and motion analysis systems make direct comparisons between our data and previous data inappropriate.

Even though liposomes improved postthaw percentages of motile spermatozoa after cryopreservation, if liposomes induced a premature acrosome reaction or inhibited a normal acrosome reaction, these motile spermatozoa might not be fertile. Therefore, it was important to determine the effects that PSCH liposomes had on other spermatozoal membrane compartments, specifically the spermatozoal acrosome. Liposomes did not alter the capacity of spermatozoa to undergo a PC12-induced acrosome reaction. These results support previous data that PSCH liposome addition did not affect the fertility of stallion spermatozoa cooled to 5°C in SMEY for 24 h prior to insemination (18) nor do PSCH liposomes fuse with bull spermatozoa (1, 33). Such fusion could alter membrane composition and affect the ability of the spermatozoa to undergo the acrosome reaction. Therefore, interactions between the spermatozoal membrane and the PSCH liposomes do not affect the ability of the plasma membrane to fuse with the spermatozoal outer acrosomal membrane.

Differences have been reported in the abilities of fresh and frozen–thawed bull spermatozoa to undergo an acrosome reaction in response to PC12 treatment and subsequently penetrate zona-free hamster oocytes (12, 13). However, we found no difference in the concentration of PC12 required to induce the acrosome reaction in fresh or frozen–thawed stallion spermatozoa. Neither did we see a difference in the relative percentage of fresh or frozen–thawed spermatozoa that exhibited an acrosome reaction after PC12 treatment.

PC12 molecules incorporate into spermatozoal membranes by monomeric transfer altering membrane organization leading to the acrosome reaction (26). Previous reports indicated that PC12 induced the acrosome reaction in both fresh and frozen–thawed spermatozoa in a dose-dependent manner (12–14). However, frozen–thawed bull spermatozoa required 10.5 μM more PC12 to induce maximal percentages of acrosome-reacted spermatozoa than fresh spermatozoa (13). The reason that frozen–thawed spermatozoa required higher levels of PC12 than fresh spermatozoa may result from PC12 incorporation into the added egg yolk as well as the spermatozoal membranes (12). The reason frozen–thawed bull and stallion spermatozoa responded differently to PC12 may result from a higher percentage of egg yolk used in the freezing medium for bull spermatozoa (20%) than for stallion spermatozoa (3%). Although the majority of the egg yolk would be removed during washing of spermatozoa prior to PC12 addition, it is likely that more egg yolk lipids remained in the bull spermatozoal treatments than the stallion spermatozoal treatments. This would necessitate higher PC12 concentrations to induce the acrosome reaction for frozen–thawed spermatozoa compared to fresh spermatozoa.

Although treatment of spermatozoa with PSCH liposomes prior to freezing in the CO medium resulted in higher postthaw percentages of motile spermatozoa than spermatozoa frozen–thawed in CO medium without liposome treatment, there were no differences detected between the two treatments in the per-

centage of viable cells, when cells were analyzed by flow cytometry ($P > 0.05$). It may be that PSCH liposomes do not actually protect spermatozoa from plasma membrane rupture during cryopreservation, but help the spermatozoon that survive freezing and thawing to maintain their motility after cryopreservation.

CONCLUSION

PSCH liposomes enhanced the percentage of motile stallion spermatozoa after cryopreservation in media containing egg yolk and skim milk and resulted in higher postthaw spermatozoal velocities than a medium containing lipid sources other than liposomes. In addition, the ability of PC12 to induce an acrosome reaction in stallion spermatozoa was not affected by either cryopreservation of the cells or addition of liposomes to the spermatozoa. Flow cytometric evaluation of cryopreserved spermatozoa, assessing multiple membrane compartments, revealed that PSCH liposomes did not alter the number of cells surviving cryopreservation. Therefore, it seems probable that liposomes help maintain motion capabilities of cells that survive cryopreservation.

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ELSEVIER

PRODUCTION OF PIGLETS PRESELECTED FOR SEX FOLLOWING IN VITRO FERTILIZATION WITH X AND Y CHROMOSOME-BEARING SPERMATOZOA SORTED BY FLOW CYTOMETRY

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ABSTRACT

In vivo-matured porcine oocytes were fertilized in vitro with X and Y chromosome-bearing spermatozoa, and sorted for sex on the basis of DNA content by flow cytometry. Developmental competence of the sexed embryos was determined through established pregnancies after embryo transfer. Spermatozoa were stained with Hoechst 33342 and sorted using a flow cytometry cell sorter. Purity of sorting was 83% for Y spermatozoa and 92% for X spermatozoa. A total of 387 mature cumulus-oocyte-complexes (COC) was collected from 18 superovulated prepuberal gilts shortly before ovulation. In vitro fertilization with sorted spermatozoa was performed in 4 replicates. After 18 h of sperm- oocyte co-culture at 39°C, the zygotes were placed into culture medium (NCSU-23) for another 24 h. The average cleavage rate was 56.2%. Ninety-two embryos produced from X-sorted sperm cells were transferred surgically into the uterus of 2 recipients. Two gilts farrowed and delivered 6 and 4 healthy female piglets, respectively. Additionally, 2 gilts were inseminated intratubally via surgical laparotomy with either X or Y sorted spermatozoa ($2x10^5$) per oviduct. The 2 sows farrowed producing 15 piglets. Thirteen of the 15 piglets were of the predicted gender (85%).
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INTRODUCTION

Economical and ecological perspectives with respect to livestock industry demand optimal herd management. Presently, maximum utilization of facilities has been achieved and sex related diseases can be avoided altogether or successfully treated, and genetic progress is being made with both male and female animals. In past years, various attempts were made to sort sperm populations based on their sex determining characteristics Through the use of flow cytometry, sperm sorting (Beltsville Sperm Sexing Technology; BSST) gender preselection in mammals can now be realized (4,5,7). Offspring have been produced after surgical insemination in rabbits (7) and swine (4), after in vitro fertilization (IVF; 1,2) or deep uterine insemination in the bovine

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(14). Earlier attempts to use IVF in the pig with sorted X and Y sperm cells (11) met with limited success. The objective of this study was to test an improved IVF protocol in conjunction with spermatozoa sorted by flow cytometry where the sorting process was appropriately synchronized to the IVF protocol. Embryo development in vitro, surgical insemination, and embryo transfer with the subsequent birth of piglets were chosen as the mean to measure the effectiveness of these procedures.

MATERIALS AND METHODS

Semen Collection and Flow Cytometry

Semen was collected from 2 boars of proven fertility. These boars were the result of a gilt and boar mating in which both animals had been produced from spermatozoa sorted for sex by the BSST system (4). The sperm rich fraction was diluted with pre-warmed Beltsville TS (BTS; 1:2, v/v; 6) immediately after collection and was evaluated for motility at x 400 magnification with a phase-contrast microscope. Sperm concentrations were determined using an improved Neubauer counting chamber (Clay-Adams, NY). Aliquots of $15x10^6$ spermatozoa were diluted in 1ml BTS (0.1% BSA). Hoechst 33342 fluorochrome (Calbiochem, La Jolla, CA) was added to achieve a final concentration of 6.25 µM, and samples were incubated for 20 min at 32° C in 1.5-ml Eppendorf tubes (4,7). Sperm cells were sorted according to the procedure described by Johnson et al. (7), using a modified EPICS V/750 series flow cytometer cell sorter (Coulter Corporation, Miami, FL; 8) at 150 mW ultraviolet (UV) light (351 and 364 nm; 5-watt 90-5 Innova Argon-ion laser, Coherent Inc., Palo Alto CA). The sorting flow rate was adjusted to 2,300 sperm/sec, for a sorting rate of 70 to 110 sperm/sec. The PBS supplemented with 0.1% BSA (Sigma, St. Louis, MO) and 2.5% EDTA (Sigma) was used as sheath-fluid, which was prefiltered through a 0.2-µm filter (Nalgene, NY). Presiliconized microcentrifuge tubes (0.6 ml, Intermountain Scientific, Kaysville, UT) used for collection were coated with BSA by filling, soaking and decanting a 1% BSA solution (7). The sort collection media was TEST extender containing 2% hen egg yolk rather than the standard 20% (4). A total of 60 µl was placed in each sort collection tube prior to sorting. Approximately $3.5x10^5$ to $4x10^5$ sperm cells were sorted into each tube after 1.5 h. The sorted semen samples were centrifuged at 800 x g for 5 min, and the remaining sperm pellet was resuspended with 200 µl fertilization medium (TALP).

In Vitro Fertilization

Eighteen prepuberal gilts, 165 d of age and averaging 76 kg, were superovulated with 1500 IU PMSG (Brumegon, Hydrochemie, Oberschleißheim, Germany), followed by 750 IU hCG (Ovogest, Hydrochemie, Oberschleißheim, Germany) 72 h later. The gilts were slaughtered 38 h after hCG treatment, and their reproductive tracts were transported in a prewarmed thermos container (38°C) to the laboratory within 5 min of slaughter. The ovaries were placed into Dulbecco's PBS (Sigma), supplemented with penicillin (Sigma 0.06 g/l) and streptomycin (Sigma 0.05 g/l). Mature follicles were punctured with an 18-gauge needle, connected to a 1-ml syringe and prefilled with

0.1 ml flushing medium. Aspirates were individually placed into Petri dishes and thereafter were collected into a 35-mm Petri dish containing 2 ml flushing medium. Cumulus-oocyte complexes (COC) were evaluated for cumulus expansion and morphological integrity of the cytoplasm. Only COC with maximally expanded cumulus cell layers and evenly granulated cytoplasm were used for the experiments. Subsequently, the COC were placed into 1 ml of fertilization medium, and $4x10^5$ sorted sperm cells/ ml were added to the culture dish. Sorted X spermatozoa were used for IVF, and were co-cultured with the COC for ~18 h. Thereafter, zygotes were washed 5 times and then transferred into the culture medium North Carolina State University (NCSU 23; 9) for another 24 h. Developing embryos (2 to 8 cell stages) were transferred to 0.25 ml modified French straws (12) for transfer to the surgery room.

Transfer of IVF Embryos

Two cyclic crossbred gilts with 3 known previous estrous cycles were synchronized with the oocyte donors at Day 17 to 18 of the cycle with a single injection of 750 IU hCG, making the recipients and donors asynchronous by minus 24 h, and were checked daily for estrus. For embryo transfer, the gilts were anesthetized with a combination of ketamine (Ketaset) tiletamine/zolazepam (Telazol; Fort Dodge, IA), butorphanol tartrate (Torbugesic, Fort Dodge, IA), xylazine (Rompun) and atropine (Phoenix Inc., St. Joseph, MO). The gilts were prepared for surgery in dorsal recumbency, and the ovaries and oviducts were exposed by midventral laparotomy. North Carolina State University culture medium (NCSU 23; 9) without taurine or hypotaurine and supplemented with 5.96 g/L HEPES was used as the transfer medium. A straw (12) containing embryos in transfer medium was inserted into the oviduct of the recipient gilts, and 46 embryos were pushed into the respective ampulla using a mandrin. The reproductive tract was then returned to the abdominal cavity.

Surgical Insemination

Cyclic crossbred gilts (n=2) were prepared for surgical insemination on the day of expected ovulation as described above. After surgical exposure of the reproductive tract, 100 µl TEST-yolk extender containing ~200,000 spermatozoa were placed into each oviduct by inserting a blunt 20-gauge needle through the utero-tubal junction into the isthmus of the oviduct as previously described (4). The first surgical insemination was performed with Y chromosome-bearing spermatozoa and the second surgical insemination with X-bearing spermatozoa.

RESULTS

Sperm Sorting

The average motility of the neat semen after collection and before dilution with BTS was 82 ± 3%. After sorting, sperm motility dropped slightly to 76 ± 6% and remained at this level (63 ± 10%) at least for 4 h. Even after 24 h of co-culture with the oocytes in fertilization medium 10 ± 8% of the sperm cells showed active movement. The purity of

the respective sorted population was 89% for X chromosome-bearing spermatozoa (predicted sex, Table 1) and 95% for the Y-bearing sperm cells (Table 1).

In Vitro Fertilization

A total of 387 COC (21.5 per donor) was aspirated from 424 follicles (recovery rate 91.2%). Of these, 379 COC were mature and met the required quality (maximal cumulus expansion and evenly granulated cytoplasm) for use in IVF. Average cleavage rate was 56.2% (n=213). Forty-eight hours after the start of IVF, 33 embryos were at the 2-cell stage, 74 at the 4-cell stage, and 106 at the 8-cell stage. From these, 92 cleaved embryos were transferred into 2 recipients, (46 embryos per recipient). Both gilts became pregnant and delivered litters of 6 and 4, respectively. Table 1 shows the distribution of the sex ratio and average birth weight per litter.

Surgical Insemination

Because neither of the recipients had ovulated at the time of insemination prominent mature follicles were present in both animals. After insemination, both gilts became pregnant and delivered a total of 15 piglets. Table 1 shows the distribution of the sex ratio and the average piglet weight per litter.

Table 1. Farrowing results from gilts receiving embryos purchased from spermatozoa sorted for the X chromosome and from gilts surgically inseminated with spermatozoa sorted for either X or Y chromosome

Fertilization method	Sperm cells sorted for	Litter size n	Average birth weight (kg)	Male offspring n	Male offspring %	Female offspring n	Female offspring %	Predicted sex (%)[b]
IVF 1[a]	X chromosome	6	2.1	0	0	6	100	89
IVF 2[a]	X chromosome	4	1.8	0	0	4	100	89
First surgical insemination	Y chromosome	7	1.6	6	86	1	14	95
Second surgical insemination	X chromosome	8	1.7	1	13	7	88	89

[a] 46 embryos transferred per recipient gilt.
[b] Based on re-analysis of an aliquot of sorted X or Y chromosome-bearing spermatozoa for DNA content (4,5).

DISCUSSION

Many attempts have been made during the past 50 to 60 yr to skew the natural sex ratio from the standard 50:50 to some proportion in favor of males or females depending on the desired sex. Only the sorting method based on flow cytometry (Beltsville Sperm Sexing Technology) has a proven record of accurately identifying X and Y chromosome-bearing spermatozoa, thus making it possible to shift the sex ratio to the desired gender (1,2,4,5). The method is dependent on distinguishing the DNA content of individual sperm cells and sorting the 2 populations into separate tubes (4) at a limited rate of 3 to 4 x 10^5 /h as individual sperm cells are sorted one by one. Either IVF or surgical insemination are efficient means for delivering sorted spermatozoa to the fertilization site. Surgical insemination has been shown to produce rabbits (7) and pigs (4) of the desired sex, while IVF has produced calves of the desired sex at rates up to 90% (1,2). In our earlier study (12), we produced sexed pig embryos utilizing gamete intra-fallopian transfer (GIFT).

Here we report on the birth of 2 litters of pigs produced by IVF using spermatozoa sorted for sex. It is the first report of the birth of piglets following IVF with spermatozoa preselected for sex based on the difference in X and Y chromosome-bearing sperm DNA. In our previous study (11), we showed that IVF with sorted spermatozoa was possible. However, in that study the number of embryos produced was limited and thus there was an insufficient number for embryo transfer. In the present study we improved the procedures for both IVF and sperm processing after sorting, which led to higher cleavage rates that were comparable to those of IVF with unsorted spermatozoa (13). In another earlier experiment (13) we utilized TCM 199. The TALP medium used in our present experiments seemed to be better suited for use with the TEST yolk (2%) extender that is necessary as a collection medium for sorted spermatozoa (4). Although all sorted sperm samples were centrifuged after sorting, small amounts of egg yolk are transferred with the spermatozoa to the fertilization medium. This minimal amount of egg yolk, however, did not appear to interfere with fertilization.

In our present study, cleavage rates after IVF, pregnancy rates, and litter size were nearly comparable to those of IVF with unsorted spermatozoa (10,15) or of the GIFT procedure (12). This indicates that neither sperm treatment with Hoechst 33342 fluorochrome nor the sorting procedure affected the fertilizing capacity of the sorted spermatozoa, and that the sperm-oocyte interaction was sufficiently synchronized to result in pregnancies. All offspring were healthy and the average weight was normal. No differences were found between litters derived from IVF and surgical insemination, but fewer sperm cells were needed per oocyte in the IVF system than in the surgical insemination procedure. Compared with other IVF experiments (10) using unsorted spermatozoa, the fertilization and pregnancy rates in this study were similar. Additional experiments are underway to confirm the present results.

An infection with a Pseudomonas subspecies was found in the ejaculates of 1 boar. This infection likely prevented pregnancies following 4 other transfers that were performed. The infection, however, did not affect IVF and in vitro development up to the

4-cell stage, as is indicated by the high cleavage rates (56.2%). Commercial application of IVF with sorted X or Y chromosome-bearing spermatozoa to produce piglets of a preselected sex will require improved efficiency of sexed embryo production as well as improvement in embryo transfer technology. Nonsurgical transfer, currently in the developmental stage (3), offers the potential for expanded use of gender selection in animal production.

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EXCEPTIONAL PAPER—RAPID PUBLICATION

Sex Preselection in Rabbits: Live Births from X and Y Sperm Separated by DNA and Cell Sorting

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ABSTRACT

Intact, viable X and Y chromosome-bearing sperm populations of the rabbit were separated according to DNA content with a flow cytometer/cell sorter. Reanalysis for DNA of an aliquot from each sorted population showed purities of 86% for X-bearing sperm and 81% for Y-bearing sperm populations. Sorted sperm were surgically inseminated into the uterus of rabbits. From does inseminated with sorted X-bearing sperm, 94% of the offspring born were females. From does inseminated with sorted Y-bearing sperm from the same ejaculates, 81% of the offspring were males. The probability of the phenotypic sex ratios differing from 50:50 were $p<0.0003$ for X-sorted sperm and $p<0.004$ for Y-sorted sperm. Thus, the phenotypic sex ratio at birth was accurately predicted from the flow-cytometrically measured proportion of X- and Y-bearing sperm used for insemination.

INTRODUCTION

The ability to preselect the sex of offspring of agriculturally important animals would have a significant impact on the genetics and economics of livestock production. The only established difference between X and Y chromosome-bearing sperm is the quantity of DNA in the sex chromosome. This difference can be measured in individual sperm using flow cytometric analysis (Pinkel et al., 1982b; Garner et al., 1983; Johnson and Pinkel, 1986; Johnson et al., 1987a). Numerous physical, biochemical, and immunological methods of semen treatment have been proposed for altering the sex ratio of offspring (for a recent review, see Amann, 1989). No conclusive proof of X- and Y-bearing sperm enrichment based on subsequent fertility has been demonstrated. Further, semen processed by most of the proposed physical, biochemical, or immunological methods has been tested by DNA analysis in recent years (Pinkel et al., 1985; Johnson, 1988a) without evidence of sex ratio alteration.

Flow cytometric measurement of sperm DNA has been used to determine the ratio of X and Y chromosome-bearing sperm in animal semen (Johnson and Pinkel, 1986; Johnson et al., 1987a). Flow-sorting was used to separate the X- and Y-bearing sperm of the vole (Pinkel et al., 1982a) and chinchilla (Johnson et al., 1987b). However, in these studies, preparation procedures damaged the viability of DNA. Recently, bull, boar, and ram sperm nuclei were flow-sorted into separate X and Y sperm populations with greater than 90% enrichment for X- or Y-bearing sperm, and the sorted sperm nuclei were shown to be capable of pronuclear development after microinjection into hamster eggs (Johnson and Clarke, 1988).

In this report, intact, viable rabbit sperm were flow-cytometrically separated into X and Y populations on the basis of relative DNA content. The separation of X- and Y-bearing sperm was verified by determining the sex ratio of offspring born.

MATERIALS AND METHODS

Semen Preparation and Flow Sorting

Semen was collected from two mixed breed mature bucks (12 ejaculates) by use of an artificial vagina and

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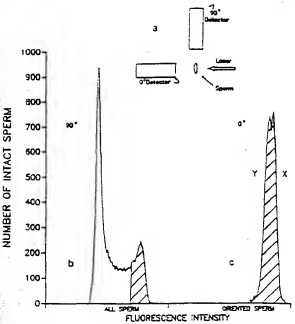

FIG. 1. a) Top view of the optical arrangement of the EPICS V Flow Cytometer/Cell Sorter (Coulter Corporation, Hialeah, FL) modified for sperm. Sample flow is perpendicular to the plane of the page. Fluorescent detectors measure fluorescence emitted in cones centered 0 and 90 degrees to the direction of the propagation of the laser beam. b) Histogram from the 90-degree detector showing the gating window (*hatched area*: 17 channels wide), selecting those sperm with their edges toward the 90-degree detector (properly oriented), and their flat faces toward the propagation of the laser beam and the 0-degree detector. c) Histogram illustrating the output from the signals captured by the 0-degree detector from the sperm falling within the gate window of the 90-degree detector.

a teaser female. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer (tris(hydroxymethyl)aminomethane, 2.52 g; D glucose, 1.04 g; citric acid, 1.28 g), pH 6.9, to a concentration of 10×10^6 sperm per ml. Bisbenzimide H33342 fluorochrome (Calbiochem-Behring, La Jolla, CA) was added to achieve a final concentration of 9 μM. The samples were incubated for 1 h at 35°C to aid penetration of the fluorochrome. Intact sperm were sorted on an EPICS V flow cytometer/cell sorter (Coulter Corporation, Hialeah, FL) modified for sperm (Johnson and Pinkel, 1986; Johnson, 1988b; Fig. 1). The stained intact sperm were excited in the ultraviolet (UV; 351 and 364 nm) lines of a 5-watt 90-5 Innova Argon-ion laser (Coherent, Inc., Palo Alto, CA) operating at 200 mW. A 76-μm jet-in-air flow tip was used. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into Test buffer (Graham et al., 1972) modified by adding 0.5% Equex STM (Nova Chemical, Inc., Scituate, MA) and 20% hen's egg yolk. Microfuge tubes (0.5 ml) served as receptacles for the sorted sperm.

To sort X- and Y-bearing intact sperm, electronic sorting windows were set on the fluorescent signal peaks obtained from the 0-degree detector measurement of the intact sperm sample (Fig. 1). Sorting windows were 10 channels wide for each of the Y and X peaks, respectively. Sorted sperm were concentrated by incubating at room temperature for 1 h, after which the more dilute upper fraction was discarded. The concentrated fraction was used for insemination 1–4 h later.

Surgical Insemination

Mature New Zealand White does were injected with 150 IU human chorionic gonadotropin (hCG) to induce ovulation, which was expected to occur 10 h later. Seven hours after treatment with hCG, the does were injected with Ketamine hydrochloride containing acepromazine, and anesthesia was maintained under halothane and oxygen. The uterus was exposed by midline incision, and 100 μl of sorted, unsorted, or recombined sperm (approximately 3×10^5) was placed into the lumen of the anterior end of each uterine horn through a 21-gauge needle. The phenotypic sex of the offspring was determined shortly after birth and was reconfirmed at various times up to 10 wk of age.

Reanalysis of Sperm for DNA Content

The relative DNA content of the sorted intact sperm used for insemination was determined by flow cytometric analysis of sperm nuclei from each of the respective fractions. Sperm nuclei were prepared from an aliquot of intact sorted sperm by sonication for 10 s in PBS and centrifuged. The sperm pellet was resuspended in PBS and Bisbenzimide H 33342 to a concentration of 9 μm (Johnson et al., 1987b). The samples of sperm nuclei were analyzed under the same instrument conditions as described above, with the exception that they were not sorted. The proportion of X- and Y-bearing sperm in nuclei samples or in sorted sperm samples was determined by fitting a pair of Gaussian distributions to the histograms from the 0-degree detector (Johnson et al., 1987b).

TABLE 1. Predicted and actual sex ratios of offspring after intrauterine insemination of sorted X and Y chromosome-bearing rabbit sperm.

Treatment of sperm	Number of does		Total no. of young born	Percentage and number of offspring			
	Inseminated	Kindled		Predicted[a]		Actual[b]	
				% Males	% Females	% Males (N)	% Females (N)
Sorted Y	16	5	21	81	19	81 (17)	19 (4)
Sorted X	14	3	16	14	86	6 (1)	94 (15)
Recombined X and Y	17	5	14	50	50	43 (6)	57 (8)
Total	47	13	51	---	---	47 (24)	53 (27)

[a]Represents the results of reanalysis for relative DNA content of aliquots of sorted X- and Y-bearing sperm populations.

[b]Represents actual births.

The data were analyzed statistically by a binomial distribution analysis (SAS Institute). The 95% confidence limits were also calculated (Narrella, 1966).

RESULTS

In an initial experiment, the fertilizing capacity of sperm was not seriously affected by the sorting process. Each of 39 ova were fertilized when recovered 40 h post-insemination from 5 does inseminated with stained, sorted sperm. All 7 ova from another doe were unfertilized. Nine ova, all fertilized, were recovered from 2 does inseminated with unstained, unsorted sperm. All fertilized ova were 8–16 cells.

The ratio of X to Y sperm in the sorted sperm fractions used for insemination accurately predicted the phenotypic sex ratio of the offspring (Table 1). Reanalysis of an aliquot of the sorted Y-bearing sperm population used for insemination indicated that 81% of the sperm were Y-bearing. The offspring from these inseminations were 81% male. Reanalysis of an aliquot of the sorted X-bearing sperm population used for insemination indicated that 86% of the sperm were X-bearing, and the offspring were 94% female. Does were also inseminated with sorted X- and Y-bearing sperm populations that were recombined immediately before insemination. From the concentration and purity of the sorted fractions, we assumed that approximately equal numbers of X- and Y-bearing sperm were present in the recombined samples. The phenotypic sex resulting from these inseminations was 57% female and 43% male (Table 1). The numbers in Table 1 include 8 young that died between 2 and 4 days of age. Those young that died were spread evenly across the three groups (3 females in sorted X, 3 males in sorted Y, and 2 females in recombined X and Y; Table 1). No phenotypic abnormalities were found. All other offspring have grown to adulthood without complication.

The probability of the observed phenotypic sex ratio results (Table 1) differing from 50:50 was $p<0.0003$ for does inseminated with X-sorted sperm and $p<0.004$ for does inseminated with Y-sorted sperm. The proportion of male and female offspring resulting from the insemination of recombined X and Y sperm did not differ from 50:50 ($p = 0.40$). These probabilities agree with those established to test against a 50:50 sex ratio for samples of similar size (Moore and Gledhill, 1988).

Figure 2 illustrates the typical analysis for relative DNA content for sperm nuclei, for intact sperm, and for sperm nuclei prepared from sorted intact sperm. The difference in DNA content between rabbit X- and Y-bearing sperm is only 3%, hence the overlap in the original histogram (Fig. 2b). The shoulders on the peaks in Figures 2c and 2d show the approximate amounts of impurity in each fraction.

Figure 3 illustrates the close relationship between the predicted percentage of male offspring and the actual offspring born. The bar for predicted percentage of males from X-sorted sperm represents a mean of 10 reanalyses of sorted sperm (14 ± 0.77%, SEM) and mean percentage of males from Y-sorted sperm (81 ± 1.90%, SEM). The 95% confidence interval (one-sided) for the proportion of females resulting from the insemination of the sorted X fraction is $0.746 \leq p \leq 1.0$, and for the proportion of males from insemination of the sorted Y fraction, $0.616 \leq p \leq 1.0$. The 95% confidence interval (two-sided) for the proportion of females when recombined X and Y sperm fractions were inseminated was $0.312 \leq p \leq 0.794$, and for males, $0.206 \leq p \leq 0.688$.

DISCUSSION

Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and

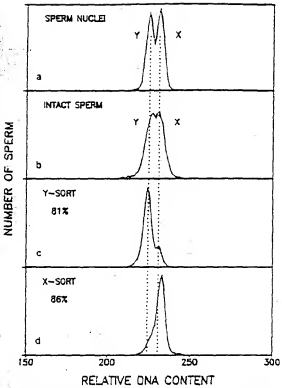

FIG. 2. Illustrates typical histogram output from the 0-degree detector for analysis and sorting of rabbit sperm. *a*) A typical frequency distribution from analysis of rabbit sperm nuclei; the calculated difference in DNA between the X and Y peaks is 3.0%. *b*) The distribution that can be obtained from intact viable sperm that are being sorted. *c*) Represents the frequency distribution obtained from reanalysis of sperm nuclei from an aliquot of sorted Y-bearing intact sperm. *d*) Represents the frequency distribution obtained from reanalysis of sperm nuclei prepared from an aliquot of sorted X-bearing, intact sperm. The *vertical dotted line* illustrates the actual relationship, in terms of relative DNA content, of the various analyses, which were all run under the same instrument conditions.

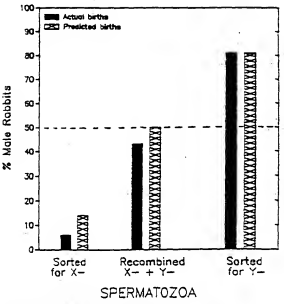

FIG. 3. Bar graph illustrating the similarity of the predicted percentages of male offspring by measuring DNA of X and Y sperm to the sex of actual offspring born.

flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently, fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head (Pinkel et al., 1982b). At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisely on 60–80% of the sperm (50–150 sperm/s) passing in front of the laser beam of the modified EPICS V flow cytometer (Johnson and Pinkel, 1986).

Intact sperm (with tails), however, whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei (Johnson, 1988b). However, the 90-degree detector can still be used to select the population of properly oriented intact sperm (Fig. 1) to be measured by the 0-degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15–20% of intact sperm pass through the laser beam with proper orientation (Fig. 1). In this study, the overall flow rate was approximately 2500 intact sperm/s. The intact X- and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80–90 X-bearing sperm/s and 80–90 Y-bearing sperm/s (Fig. 2).

Natural mating generally results in a kindling rate of 80% and a litter size of 6 young born (Casady et al., 1966). The lower kindling rate (28%) and litter size (3.9) in this study are thought to be due to reduced egg pickup because of manipulation of the uterus during

surgical insemination and the possible effects of the DNA-bound fluorochrome (unpublished data). The stage of gestation at which embryonic death may occur has not been established.

Earlier work in this laboratory has shown chromosome breakage in the developing sperm pronucleus after vole sperm nuclei were flow-sorted and microinjected into hamster eggs (Libbus et al., 1987). Those sperm, however, had been sonicated, stained, sorted and microinjected, whereas in this study the sperm were stained and sorted only. Other attempts have also been made to flow-sort sperm for insemination. These attempts have resulted in very low pregnancy rates (Morrell et al., 1988).

The results described here represent a significant advance toward the goal of sex preselection for mammals. However, several factors mitigate against widespread application of this methodology at the present time: (1) The limitation on number of sperm that can be sorted in a reasonable period of time (about 3.5×10^5 per hour) eliminates use of this procedure for producing sexed semen for standard cervical artificial insemination in most mammals. (2) The increased embryo mortality presumed to be related to the presence of the fluorochrome on the DNA. (3) The cost of the modified flow cytometer/sperm-sorting instrumentation (approximately $250,000). However, none of these factors appear to represent insurmountable difficulties. In fact, the current procedure might be effectively used in conjunction with in vitro fertilization, especially with respect to cattle.

These data provide conclusive evidence that flow-cytometrically determined sperm DNA content can be used as a differentiating marker between X- and Y-bearing sperm for purposes of determining sperm sex ratio, that measurement of DNA in separated X- and Y-bearing sperm populations can be used to predetermine the sex of offspring, and that flow-sorting is effective for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

ACKNOWLEDGMENTS

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Assessment of Ram and Boar Spermatozoa during Cell-sorting by Flow Cytometry

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Contents

The aim of this study was to improve the quality of ram and boar spermatozoa before and after the processes of 'sex-sorting' by flow cytometry. Three semen diluents are examined, hepes-buffered SOF (HSOF), Beltsville thawing solution (BTS) and phosphate-buffered saline in the absence or presence of Ca^{2+} and Mg^{2+} salts (PBS or dPBS), with and without homologous or heterologous seminal plasma. Motility and acrosome integrity of spermatozoa were observed by light microscopy and the viability was assessed by flow cytometry after dual-staining with Hoechst 33342 and propidium iodide.

The inclusion of 10% ram seminal plasma in the diluent increased motility and viability of ram spermatozoa, except in BTS where seminal plasma had no effect on viability. Boar seminal plasma increased the viability of boar spermatozoa in HSOF and motility in BTS. Heterologous seminal plasma had a negative effect on the viability of ram and boar spermatozoa but increased the motility of boar spermatozoa diluted in BTS and ram spermatozoa diluted in BTS and PBS. The presence of Ca^{2+} and Mg^{2+} salts in PBS enhanced motility and viability of ram spermatozoa but not to the same extent as ram seminal plasma.

The proportion of viable and motile spermatozoa did not always correlate. To study this further, sorted populations of 'viable' and 'dead' spermatozoa were assessed for motility and acrosome integrity. Motility, but not acrosome integrity of ram and boar spermatozoa was reduced following sorting by flow cytometry. In the 'viable' sorted population, the acrosome integrity was always high (> 72%) but motility was variable (range 10–85%). The 'dead' sorted population, however, had a low percentage of intact acrosomes (< 40%) and no motile spermatozoa.

Careful selection of a pre-sort diluent containing homologous seminal plasma and detection of viable spermatozoa while sorting on a flow cytometer could improve the quality of sex-sorted spermatozoa.

Inhalt

Bewertung von Schafbock- und Ebersperma während des ‚Cell Sorting' mit Hilfe der Flowcytometrie

Das Ziel der Untersuchung war die Verbesserung der Spermienqualität bei Schafbock und Eber vor und nach dem ‚Sex Sorting'-Prozeß mittels Flowcytometrie. Drei verschiedene Samenverdünner wurden untersucht: mit Hepes-gepufferte SOF (HSOF), Beltsville thawing solution (BTS) und Phosphatgepufferter Salzlösung mit und ohne Calcium- und Magnesium-Ionen (PBS oder dPBS), mit und ohne homologem und heterologem Seminalplasma. Dabei wurden lichtmikroskopisch Motilität und Akrosomenintegrität der Spermien bewertet. Die Lebensfähigkeit wurde zusätzlich durch flowcytometrische Messungen nach Doppelfärbung mit Hoechst 33342 und Propidiumiodid bestimmt.

Die Zugabe von 10% Schafbock-Seminalplasma zum Verdünner verbesserte die Motilität und Lebensfähigkeit der Schafbockspermien, lediglich in BTS hatte Seminalplasma keinen Einfluß auf die Lebensfähigkeit. Seminalplasma vom Eber verbesserte die Lebensfähigkeit von Ebersperma in HSOF und die Motilität in BTS. Heterologes Seminalplasma hatte einen negativen Einfluß auf die Lebensfähigkeit von Schafbock- und Ebersperma, es wurde aber eine Verbesserung der Motilität bei Ebersperma in BTS und beim Schafbocksperma in BTS und PBS gefunden. Die Ergänzung von PBS mit Calcium- und Magnesium-Salzen verbesserte die Motilität und Lebensfähigkeit von Schafbocksperma, allerdings nicht im gleichen Ausmaß wie durch Seminalplasma.

Der Anteil lebender und motiler Spermien war nicht immer korreliert. Um weitere Informationen hierzu zu bekommen, wurden gesortete Spermienpopulationen von ‚lebenden' und ‚toten' Spermien hinsichtlich Motilität und Akrosomenintegrität untersucht. Die Motilität, nicht aber die Akrosomenintegrität der Schafbock- und Ebersperma wurde durch Flowcytometrie reduziert. In der ‚lebend gesorteten' Spermapopulation war die Akrosomenintegrität immer höher (> 72%), wobei die Motilität zwischen 10 und 85% in der ‚tot gesorteten' (< 40%) schwankte und keine mobilen Spermien aufwies. Eine sorgfältige Auswahl des Verdünnermediums vor dem Sortieren, in dem Seminalplasma enthalten ist und die Bestimmung lebender Spermien während des Sortiervorganges in einem Flowcytometer können die Qualität von geschlechtsgetrennten Spermien verbessern.

Introduction

During processing for 'sex-sorting' by flow cytometry spermatozoa experience nuclear staining, exposure to a laser and high rates of dilution. Prior to sorting, ram and boar spermatozoa are extended 400 and 20-fold, respectively, and are incubated at 35°C for 1 h to allow homogenous staining by the DNA-permeant fluorescent dye Hoechst 33342 (H33342; Calbiochem, Alexandria, NSW, Australia) The spermatozoa are diluted a further 100-fold with sheath fluid during the passage through the cell sorter. Ram spermatozoa are particularly sensitive to dilution (Mann 1964). In initial studies, the motility of ram spermatozoa after sorting was 5–20% and their ability to fertilize *in vitro* matured oocytes was very low (Rhodes et al. 1994). No pregnancies were achieved after oviducal artificial insemination of 33 ewes with sex-sorted spermatozoa (Catt et al. unpublished data).

Boar spermatozoa appear to withstand the rigours of flow cytometry slightly better than ram spermatozoa (Maxwell et al. 1996). Using sex-sorted spermatozoa, piglets have been born following surgical insemination of gilts (Johnson 1991) and fertilisation of *in vitro* matured porcine oocytes can be achieved (O'Brien et al. 1995) although conception and fertilisation rates, respectively, were low.

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The viability of spermatozoa can be assessed simultaneously with sex-sorting on a flow cytometer by dual-staining the spermatozoa with H33342 and propidium iodide (PI). By selection of only live (PI negative) cells, the quality of sex-sorted spermatozoa has been improved (Johnson et al. 1994). This method of assessment was applied in this study, in conjunction with motility scoring and assessment of acrosome integrity by light microscopy, to examine the effects of three diluents with and without the addition of homologous and heterologous seminal plasma, and Ca^{2+} and Mg^{2+} salts. Pre- and post-sort populations of spermatozoa were also compared by analysis of their motility and assessment of acrosome integrity by light microscopy. A preliminary report on part of this study has been presented elsewhere (Catt et al. 1995).

Materials and Methods

Reagents and media

All chemicals were of analytical grade and unless otherwise stated were from Sigma (Castle Hill, NSW, Australia) and were made up in milli-Q® water. The constituents of the diluents used in the experiments were as follows:

PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4;

Dulbecco's PBS (dPBS): as for PBS plus 1 mM $CaCl_2$, 1 mM $MgCl_2$; BTS (Johnson et al. 1988): 205 mM D-Glucose, 20.4 mM tri-sodium citrate, 3.36 mM EDTA di-sodium salt, 14.9 mM $NaHCO_3$;

Modified hepes-buffered synthetic oviduct fluid (HSOF) (Tervit et al. 1972): 1.72 mM $CaCl_2 \cdot 2H_2O$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 7.12 mM KCl, 1.17 mM KH_2PO_4, 4 mM $NaHCO_3$, 108.5 mM NaCl, 1 mM L-glutamine, 2.4 mM D-Glucose, 10.59 mM Hepes (free acid), 10.48 mM Na Hepes, 4.19 mM Na lactate, 0.327 mM Na pyruvate, 0.3% BSA w/v (Fraction V).

Penicillin (125 IU/ml) and streptomycin (38 IU/ml) were added to all diluents and the pH and osmolarity adjusted to 7.4 and 290–300 mOsm/kg, respectively.

Seminal plasma preparation

Ram seminal plasma (rSP): Semen was pooled from 4 rams, centrifuged at 12 000 × g for 15 min, and the supernatant harvested and filtered through a 0.22 μm filter (Acrodisc, Gelman Sciences, Lane Cove, NSW, Australia). Boar seminal plasma (bSP): Seminal plasma collected from the sperm poor fraction from 3 boars was pooled, centrifuged at 12 000 × g for 15 min and the supernatant harvested.

SP preparations were stored at -20°C until required. They were added to the diluents, where required, at 10% (v/v). All diluents were filtered through a 0.22 μm filter after the addition of SP.

Collection and dilution of semen

Ram semen was collected by artificial vagina, allowed to reach room temperature and assessed for motility and concentration. Boar semen was collected at a commercial artificial insemination centre (Pig Improvement Centre, Grong-Grong, NSW, Australia) by the gloved-hand method, diluted 5-fold with BTS, transported and stored for up to 24 h at 18°C. Boar semen was allowed to reach room temperature and then assessed for motility and concentration. Semen with an initial motility > 70% was diluted to 10 × 10^8 spermatozoa/ml (≈ 400-fold and 20-fold dilution for ram and boar semen, respectively) with the appropriate diluent.

Assessment of spermatozoa by microscopy

Motility

The percentage of motile spermatozoa was assessed (at 37°C) under a coverslip using phase-contrast microscopy (100 ×).

Acrosome integrity

A drop of diluted semen was smeared on a slide and allowed to air dry. Smears were stained with Naphthol Yellow S (Sigma) and Erythrosin B (Sigma) as first described by Bryan and Akruk (1977), except that the slides were in the dual-stain for 14 min. Slides were examined by phase contrast microscopy using a 100 × oil immersion objective and white light. Spermatozoa (200 per slide) were assessed for the percentage of intact acrosomes.

Assessment and sorting of spermatozoa by flow cytometry

The extended spermatozoa were stained with H33342 essentially as described by Johnson et al. (1989), except that 8 μm H33342 was added instead of 9 μm (from a 0.5 mg/ml stock solution in water) and incubation was for 1 h at 35°C. Stained sperm samples were then held at 30°C until assessment. Five minutes prior to analysis, all samples were stained with 10 μg/ml PI (from a stock of 1 mg/ml in water). Spermatozoa were analysed and where required, sorted on a modified FACStar cell sorter (Becton-Dickinson, Ryde, NSW, Australia), with modifications first described by Johnson and Pinkel (1986). The sorter was fitted with two fluorescence detectors at right angles to each other (Fl-2 detector, parallel to, and Fl-1 detector perpendicular to the laser beam) and a bevelled sample injection needle to aid orientation. A 5 watt 90–5 argon-ion laser (Coherent Scientific, Hilton, SA, Australia) operated at 125 mW, exciting the sample in the UV range with emitted fluorescence detected through 425 band pass filters. For viability analysis, 10 000 events per sample were collected as 256 channel histograms. The PI stain, taken up by spermatozoa with damaged membranes (dead population), quenches the H33342 stain thereby producing less fluorescence. The intact (viable) and dead populations were measured as a percentage of the total events. The agglutinated spermatozoa (i.e. events collected outside and above the range of the two peaks) were not classed as viable in this analysis. The viable, dead and agglutinated populations were gated and percentages calculated using Consort 1 software (Hewlett-Packard, North Ryde, NSW, Australia) by analysis of the histogram from events collected through the FL-2 detector. For sorting, the populations were gated on a dot plot collecting data from detectors FL-1 and FL-2. The populations of spermatozoa were sorted onto glass slides and immediately analysed for

SCIENCE REFERENCE
4 MAR 1998

motility or immediately smeared and dried for analysis of acrosome integrity. PBS was the sheath fluid used in all the experiments.

Statistical Analyses

The percentage viability and motility data from experiments 2–5 were subjected to a split plot analysis of variance with diluent, rams (experiments 2 and 3), boars (experiments 4 and 5) and replicate being the main plots and time being the subplot. The percentage viability and motility data from experiment 6 were subjected to a split plot analysis of variance with diluent and rams being the main plots and time being the subplot. The percentage motility and intact acrosomes from experiments 7 and 8 were subjected to a split plot analysis of variance with diluent, rams (experiment 7), boars (experiment 8) being the main plots and type (pre- and post-sort) being the subplot. Significant differences between individual treatment means were determined by the least significant difference procedure. All analyses were performed using MINITAB (Release 10.1 1994; Minitab Inc., Pennsylvania, USA) and where appropriate (experiments 2–5), error mean squares were calculated manually.

Experimental Design and Results

Acrosome integrity, motility and viability of sorted ram and boar spermatozoa

Experiment 1

Two samples of diluted boar and three samples of diluted ram spermatozoa were sorted onto microscope slides into four populations: total sort, viable, dead and agglutinated, and were assessed microscopically for motility and acrosome integrity as previously described. Due to the high dilution rate following sorting, motility was scored without a coverslip.

Results are presented in Table 1. Following sorting, the percentage of spermatozoa with intact acrosomes was highest in the viable sort (mean 87.7% for ram, mean 73% for boar) and lowest in the dead sort (mean 35.3% for ram, mean 17% for boar). The agglutinated sort also had a high percentage of intact acrosomes (mean 70.7% for ram, mean 64.5% for boar). No motile spermatozoa were observed in the dead sort, but post-sort motility of spermatozoa in the total, viable and agglutinated sorts was variable (range 10–85% for ram and 5–60% for boar). Post-sort agglutination (observed visually) was rare. It was noted, particularly in one boar sample and one ram sample, that spermatozoa could lose motility while retaining acrosome integrity and viability.

Effect of homologous and heterologous seminal plasma on the viability and motility of diluted ram spermatozoa

Two factorial experiments (3 × 3 × 3 × 3 × 2) were performed on ram spermatozoa in order to establish the best diluent to use prior to sorting through a flow cytometer. The semen from three rams were diluted with PBS, HSOF or BTS with and without the addition of 10% seminal plasma (experiment 2, rSP and experiment 3, bSP). Samples of spermatozoa were analysed for motility and viability immediately after incubation with H33342 (time 0 h) and after storage at 30°C for a further 2 and 4 h. Each experiment was replicated three times using ejaculates from the same animals.

Experiment 2

The inclusion of rSP in all three diluents increased motility (Fig. 1a) and viability (Fig. 1c) of ram spermatozoa, except in BTS where rSP had no effect on viability ($p < 0.001$). Overall, there was a reduction in the percentage of viable and motile spermatozoa over the 4 h holding period, but the decline in motility was greatest for spermatozoa diluted with HSOF-rSP and BTS-rSP (Fig. 2a) and the decline in viability was greatest for HSOF-rSP (Fig. 2c).

Experiment 3

Boar SP increased motility of ram spermatozoa extended in BTS and PBS ($p < 0.01$, Fig. 1b) and decreased the viability of spermatozoa in HSOF ($p < 0.01$) and BTS ($p < 0.001$, Fig. 1d).

Effect of homologous and heterologous seminal plasma on the viability and motility of diluted boar spermatozoa

The two factorial experiments performed on ram spermatozoa were repeated using boar spermatozoa. The semen from three boars were diluted with PBS, HSOF or BTS with and without the addition of 10% seminal plasma (experiment. 4, rSP and experiment. 5, bSP).

Table 1. Percent motile and viable spermatozoa and spermatozoa with intact acrosomes (% intact) pre-sort and % motile and intact spermatozoa after sorting onto microscope slides into four populations: total sort, viable, dead and agglutinated (Agg)

			Ram				Boar		
Diluent			PBS + rSP	BTS	BTS + rSP	Mean	BTS	BTS + bSP	Mean
Pre-sort	Total	% intact	95	75	48	73	79	48	64
		% motile	75	55	60	63	30	60	45
		% viable	54	50	49	49	70	47	59
Post-sort	Total sort	% intact	71	65	53	63	68	67	68
		% motile	75	25	30	43	10	40	25
	Viable	% intact	88	84	91	88	74	72	73
		% motile	85	60	10	52	15	60	38
	Dead	% intact	38	39	29	35	12	22	17
		% motile	0	0	0	0	0	0	0
	Agg	% intact	78	65	69	71	59	70	65
		% motile	70	60	20	50	5	40	23

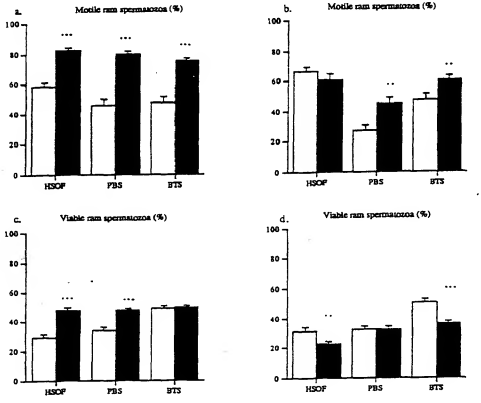

Fig. 1. Motility (top) and viability (bottom) of diluted ram spermatozoa, following incubation in diluent without (open bars), with homologous (a and c, closed bars), or with heterologous seminal plasma (b and d, closed bars). Values are means ± SEM for all three evaluation times. Paired values differ * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.0001$)

Experiment 4

Boar SP increased the motility of boar spermatozoa when included in BTS and viability when included in HSOF ($p < 0.05$, Fig. 3a and 3c, respectively). Boar SP had no effect on motility and viability when included in the other diluents. There was a reduction in the percentage of motile spermatozoa over the 4 h holding period and the decline in motility was relatively constant between treatments (Fig. 2b). Overall, motility was best for HSOF and PBS containing SP. After 4 h the viability of boar spermatozoa for all diluents remained high (range of means 52–64%, Fig. 2d) and was best in HSOF containing SP.

Experiment 5

Viability of boar spermatozoa was lower in the presence of rSP than in its absence ($p < 0.001$, Fig. 3d) and motility was enhanced by rSP in BTS only ($p < 0.05$, Fig. 3b). The decrease in viability in the presence of rSP was not due to an increase in agglutination which varied little between diluents (range of means 28.1–33.8%).

Effects of Ca^{2+} and Mg^{2+} salts and homologous seminal plasma on the viability and motility of ram spermatozoa diluted in PBS

Experiment 6

A factorial experiment (3 × 3 × 3; diluents × rams × time) examined whether Ca^{2+} and Mg^{2+} salts could be substituted for homologous seminal plasma. Ejaculates from three rams were split and diluted with PBS containing Ca^{2+} and Mg^{2+} salts (dPBS), PBS or PBS + rSP. Aliquots of spermatozoa from each dilution were analysed for motility and viability immediately after incubation with H33342 (time 0 h) and after storage at 30°C for a further 2 and 4 h.

Results are presented in Fig. 4a and b. Dilution with dPBS compared to dilution with PBS had no effect on the motility but increased viability ($p < 0.05$) of ram spermatozoa. Viability and motility of spermatozoa diluted with dPBS was less than that diluted with PBS + rSP ($p < 0.05$).

Pre- and post-sort assessment of acrosome integrity and motility of spermatozoa

Experiments 7 *and* 8

Two factorial experiments (3 × 3 × 2) were conducted to compare acrosome integrity and motility of ram and boar spermatozoa pre- and post-sort. Ejaculates from three rams and three boars were split and diluted with PBS, BTS, BTS + rSP (rams, experiment 7) or BTS + bSP (boars, experiment 8). Sub-samples of spermatozoa from each dilution were analysed for motility and slides prepared for analysis of acrosome integrity following 1 h incubation with H33342 at 35°C. The remaining samples were sorted onto microscope slides, by gating the total population, and immediately assessed microscopically for motility and acrosome integrity of spermatozoa.

There was no decrease in post- compared to pre-sort acrosome integrity (64.1 ± 2.81 vs. 70.1% ± 3.37 for ram, 43.3 ± 6.95% vs. 49.2 ± 4.97% for boar) but there was a decrease in motility post- compared to pre-sort (31.1 ± 7.98% vs. 66.7 ± 4.86%, $p < 0.001$ for ram and

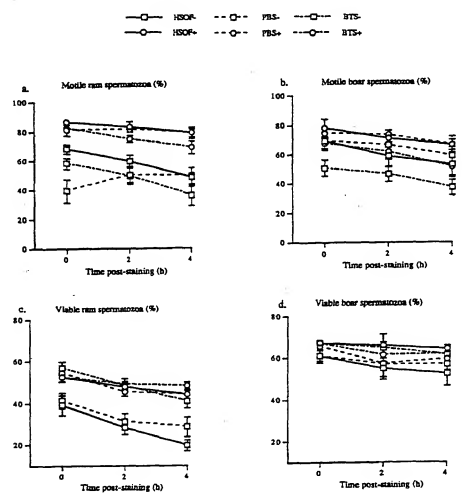

Fig. 2. Effect of diluent and time on the motility and viability of ram (a and c), and boar (b and d) spermatozoa, respectively, diluted with HSOF, PBS and BTS, without (–), or with (+) homologous seminal plasma. Values are means ± SEM

47.8 ± 5.47% vs. 68.3 ± 4.64%, $p < 0.01$ for boar). Rams (experiment 7), boars (experiment 8) and diluents (experiments 7 and 8) had no influence on motility or acrosome integrity of the spermatozoa.

Discussion

Separate studies have been performed on viability (Ashworth et al. 1994) and motility (Blackshaw 1953; Harrison et al. 1982) of ram spermatozoa after high dilution rates. It is apparent from this study that these indicators of sperm quality do not always correlate. Assessment of viability by flow cytometry has the advantage that many thousands of spermatozoa can be analysed in a few seconds, compared to microscopic assays of viability, motility and acrosome integrity where it is only practical to analyse up to 200 spermatozoa per slide. However, 'viable' spermatozoa are sometimes assessed to be immotile and it is impossible to determine the viability of agglutinated spermatozoa. Also up to 40% of motile ram spermatozoa have been classed as 'non-viable' (see Fig. 1). Maxwell et al. (1996), using similar methodology, reported motility scores higher than viability scores for the same populations of ram and bull but not boar spermatozoa. Therefore, it seems necessary to support the viability assay with assessment of motility and vice-versa.

The findings of this study were consistent with that of Ashworth et al. (1994), in that the motility and viability of ram spermatozoa deteriorated when the semen was diluted in a simple salt solution, and this deterioration could be lessened by the addition of 10% ram seminal plasma to the diluent. Motility was also reduced when ram spermatozoa were extended with the more complex media: HSOF and BTS. However, when these diluents contained 10% ram seminal plasma, the extended spermatozoa retained pre-dilution levels of motility (70–80%). The highest proportion of viable ram spermatozoa was obtained when the semen was diluted in BTS compared with HSOF and PBS, but viability of spermatozoa was improved if the latter two diluents contained ram seminal plasma. The improved viability of spermatozoa in the presence of ram seminal plasma was partially, but not totally due to a decrease in the proportion of 'agglutinated' cells.

Dilution of boar spermatozoa to a concentration of 10 million sperm/ml did not cause the dramatic loss of viability and motility seen with ram spermatozoa. Moreover, during the 4 h incubation period after staining more than 50% of spermatozoa remained viable. This may be simply due to a lower overall rate of dilution required for boar (≈ 1 in 20) compared with ram (1 in 400) semen. In

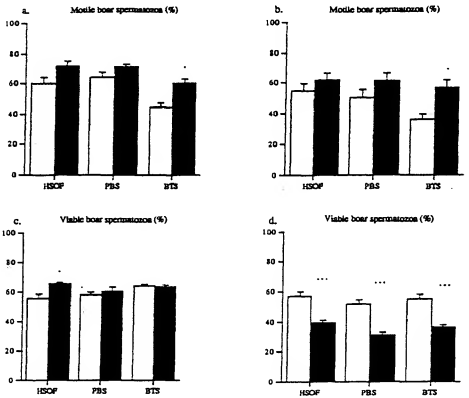

Fig. 3. Motility (top) and viability (bottom) of diluted boar spermatozoa, following incubation in diluent without (open bars), with homologous (a and c, closed bars), or with heterologous seminal plasma (b and d, closed bars). Values are means ± SEM for all three evaluation times. Paired values differ * ($p < 0.05$), ***($p < 0.0001$)

the former case, the low rate of dilution may not have been enough to eliminate the protective factors in seminal plasma. Moreover, the commercial boar spermatozoa had already been diluted five-fold with BTS prior to the overnight storage and delivery to the laboratory. It must be taken into account therefore that all diluents would partially contain the constituents of BTS. Nevertheless, the motility of boar spermatozoa in BTS alone was lower than for semen extended with HSOF or PBS, or compared to BTS with the addition of boar or ram seminal plasma. This was surprising since BTS is a widely used commercial boar semen diluent.

In general, heterologous seminal plasma reduced the viability of diluted ram and boar spermatozoa, and hence had quite the opposite effect of adding homologous seminal plasma. It was also noticed that heterologous seminal plasma caused a loss of resolution between the live and dead populations. Leading up to fertilization a cascade of events occur on the sperm surface involving both non-specific and species-specific protein binding sites and these have been extensively reviewed by Fraser and Ahuja (1988) and Miller and Ax (1990). It is possible that proteins from the heterologous seminal plasma may compete with existing species-specific proteins for binding and cause a destabilised state on the sperm membranes. Motility of ram spermatozoa was improved by adding heterologous seminal plasma to spermatozoa diluted in PBS and BTS, but not to the same extent as homologous seminal plasma. Motility of boar spermatozoa was improved by adding heterologous seminal plasma to all diluents and the improvements were to a similar level as homologous seminal plasma. Calvette et al. (1995), on reviewing the mode of action of sperm surface proteins, speculated that seminal plasma contains positive and negative regulatory factors, affecting the process of capacitation and/or sperm transport.

The composition of seminal plasma, made up of inorganic compounds, amino-acids, peptides, low and high molecular weight proteins, varies depending on species (Rodger 1975), interval between ejaculations (Strzezek et al. 1995) and health of the animal (Lynch et al. 1994). Our finding that BTS could maintain sperm viability with or without the addition of seminal plasma suggests that simple metabolites or ionic components, other than proteins, could substitute for seminal plasma in the maintenance of cell viability. Previous reports (Ashworth et al. 1994) have shown that the replacement of most of the NaCl in a simple saline medium with an energy source (sucrose) increased viability and it is possible that glucose (a constituent of BTS) may play a similar role. Glucose is also present in HSOF but at levels $\approx 100\times$ less than in BTS. Bredderman and Foote (1971) found that EDTA, a constituent of BTS, could stabilise bull sperm membranes.

The principal aim of this study was to increase the quality of flow-sorted spermatozoa. The addition of homologous seminal plasma to pre-sorted semen increased viability and motility particularly of ram spermatozoa. An additional problem remains that, during flow sorting, the spermatozoa are further diluted with the sheath fluid. The majority of sheath fluid flows to waste and ≈ 2 l is used per 2 h sort. PBS has been commonly used as sheath fluid and since it would be impractical to collect up to 200 ml of seminal plasma to add to this, the possibility of

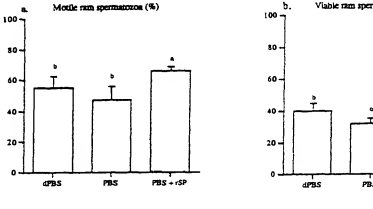

Fig. 4. Effect of additional salts and homolgous seminal plasma on the motility (a) and viabilty (b) of ram spermatozoa, diluted with PBS. Values are means ± SEM for all 3 evaluation times. [a,b,c] ($p < 0.05$), [a] ($p < 0.001$)

simply including additional salts to PBS was explored. Calcium and magnesium salts improved viability and motility of ram spermatozoa when added to PBS (dPBS) compared to PBS alone, but not to the same extent as ram seminal plasma.

No significant loss in acrosome integrity was observed as a result of flow cytometric sorting of spermatozoa. However, motility was lower for both ram and boar post-sort compared with pre-sort spermatozoa. Because spermatozoa were sorted directly onto slides, the loss in motility may be due to dilution alone. During normal sorting, it is now the authors' practice to sort onto a cushion of medium such as 50% v/v homologous SP in PBS (Catt et al. 1996) or test-yolk buffer (O'Brien et al. 1995) and this has been shown to maintain the motility of spermatozoa post-sort (Maxwell et al. 1996).

Based on the results of this study, the addition of ram seminal plasma to any of the diluents tested can be recommended for pre-sort dilution of ram spermatozoa, but if ram seminal plasma is unavailable then BTS would be the preferred diluent. HSOF with boar seminal plasma was the best pre-sort diluent for boar spermatozoa. Further work is now required to determine whether improved sperm quality following sex-sorting leads to improved fertilisation rates *in vitro* and *in vivo*.

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[54] **METHOD TO PRESELECT THE SEX OF OFFSPRING**

[75] Inventor: **Lawrence A. Johnson**, Silver Spring, Md.

[73] Assignee: **The United States of America as represented by the Secretary of Agriculture**, Washington, D.C.

[21] Appl. No.: **692,958**

[22] Filed: **Apr. 26, 1991**

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[63] Continuation of Ser. No. 349,669, May 10, 1989, abandoned.

[51] Int. Cl.[5] **A61K 35/52**

[52] U.S. Cl. **424/561**; 436/63; 436/172; 435/2

[58] Field of Search 436/63, 172; 424/561

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[57] **ABSTRACT**

Intact X and Y chromosome-bearing sperm populations of rabbits and swine were separated according to DNA content using a flow cytometer/cell sorter. Sperm viability was maintained by special staining techniques and by sorting and collecting the sperm in nutrient media. The sorted sperm were surgically inseminated into the uteri of rabbits or swine. Of the offspring born from does inseminated with the sorted population of X-bearing sperm, 94% were females. Of offspring born from does inseminated with sorted Y-bearing sperm from the same ejaculate, 81% were males.

26 Claims, No Drawings

METHOD TO PRESELECT THE SEX OF OFFSPRING

This application is a continuation of application Ser. No. 07/349,669, filed May 10, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method of preselecting the sex of offspring by sorting sperm into X and Y chromosome-bearing sperm based on differences in DNA content.

2. Description of the Prior Art

Gender of animal offspring is important to livestock producers. Because the dairy farmer has little use for most bull calves, the use of sexed semen to produce only females would make milk production more efficient. Swine farmers would produce pork more efficiently if they were able to market only female swine, because females grow faster than males.

In beef cattle and sheep breeds, the male grows at a faster rate than the female and hence is preferred for meat production.

In addition, the ability to specify male or female offspring should shorten the time required for genetic improvements, since desirable traits are often associated with one or the other parent. Planning the sex of cattle offspring is already practiced on a limited basis. This procedure consists of removing embryos from the cow, identifying their potential gender, and re-implanting only those of the desired gender. However, an ability to separate sperm into male-producing and female-producing groups before they are used for artificial insemination could enhance the overall value of offspring produced by embryo transfer.

Every living being has a set of paired chromosomes, which carry all the genetic material necessary to maintain life and also to propagate new life.

All but one pair of chromosomes are called autosomes and carry genes for all the characteristics of the body, such as skin, hair and eye color, mature size, and body characteristics. The remaining pair are called sex chromosomes. They carry the genetic material that specifies gender. One sex chromosome is called X, the other Y.

A sperm from the male or an egg from the female contains one of each pair of autosomes; in addition, in mammals the egg always contains an X chromosome, while the sperm always carries either an X or Y chromosome.

When a sperm and egg unite and the sperm carries the Y chromosome, the offspring is male (XY); however, if the sperm carries an X chromosome when it unites with the egg, the resulting offspring is female (XX).

The only established and measurable difference between X and Y sperm that is known and has been proved to be scientifically valid is their difference in deoxyribonucleic acid (DNA) content. The X chromosome is larger and contains slightly more DNA than does the Y chromosome. The difference in total DNA between X-bearing sperm and Y-bearing sperm is 3.4% in boar, 3.8% in bull, and 4.2% in ram sperm.

The amount of DNA in a sperm cell, as in most normal body cells, is stable. Therefore, the DNA content of individual sperm can be monitored and used to differentiate X- and Y-bearing sperm.

Since the difference in DNA mass in the sex chromosomes of most mammals is the only scientifically validated, measurable difference between X- and Y-bearing sperm, the chromosomal constitution [Moruzzi, J. Reprod. Fertil. 57: 319 (1979)] and/or measurement of DNA mass [Pinkel et al. (1), Science 218: 904 (1982); Pinkel et al. (2), Cytometry 3: 1 (1982); Johnson and Pinkel, Cytometry 7: 268 (1986); Johnson et al. (1), Gam. Res. 16: 1 (1987); Johnson et al. (2), Gam. Res. 17: 203 (1987)] are the only verifiable means other than fertility for determining the sex-producing capability of a population of sperm. The literature describes many physical, biochemical, and functional methods that have purportedly sexed sperm [Amann and Seidel, "Prospects for Sexing Mammalian Sperm," Colorado Assoc. Univ. Press, Boulder (1982)]; several of these methods have been tested for relative DNA content [Pinkel et al., J. Anim. Sci. 60: 1303 (1985); Johnson (1), Theriogenolgy 29: 265 (1988)]. However, no method has been proven in controlled experiments to actually affect the sex ratio of offspring.

Previous studies have demonstrated that the difference in DNA content between X and Y chromosome-bearing sperm can be repeatedly measured and the sperm sex ratio of a sample of semen predicted [Johnson and Pinkel, supra; Johnson et al. (1), supra; Johnson et al. (2), supra; Johnson (1), supra; Johnson (2), Cytometry, Suppl. 2: 66 (Abstract) (1988)]. Verifiable separation by sorting of X and Y sperm based on DNA content has been accomplished with the vole [Pinkel et al. (1), supra; Johnson, In "Beltsville Symposia in Agricultural Research X," P. C. Augustine, H. D. Danforth, & M. R. Bakst (eds.), Martinus Nijhoff, Boston, pp. 121-134 (1986)] and the chinchilla [(Johnson et al. (1), supra]. However, preparation procedures damaged DNA viability. The sorting of sperm nuclei from several mammalian (bull, boar, ram, vole, chincilla) species into separate X and Y chromosome-bearing populations at purities ranging from 92 to 99% has been accomplished [Johnson and Clarke, Gam. Res. 21: 335 (1988)]. Nuclear decondensation and pronuclear development was demonsrrated in hamster eggs that had been microinjected with sorted X- or Y-bearing bull, boar, or ram sperm [Johnson and Clarke, supra].

SUMMARY OF THE INVENTION

It is an object of this invention to provide a method for sorting mammalian sperm into X and Y chromosome fractions based on DNA content.

It is a further object of this invention to teach a method of staining the DNA of mammalian sperm while maintaining viability of the sperm.

It is a further object of this invention to provide a sheath fluid adapted to be used in a cell-sorting apparatus while maintaining viability of sperm cells.

It is a further object of this invention to provide a collecting fluid capable of maintaining the viability of sorted sperm cells.

Other objects and advantages of this invention will become readily apparent from the ensuing description.

DETAILED DESCRIPTION OF THE INVENTION

I have now demonstrated the separation, by flow sorting, of intact, viable X and Y chromosome-bearing rabbit and swine sperm populations based on relative DNA content; surgical insemination of the sorted sperm into does; and the subsequent birth of sexed offspring

with a phenotypic sex ratio consistent with predictions based on the relative DNA content of the sorted sperm populations.

A flow cytometer measures the amount of fluorescent light given off when the sperm, previously treated with a fluorescent dye, pass through a laser beam. The dye binds to the DNA. The fluorescent light is collected by an optical lens assembly; the signal is transported to a photomultiple tube, amplied, and analyzed by computer. Because the X chromosome contains more DNA than the Y chromosome, the female sperm (X) takes up more dye and gives off more fluorescent light than the male sperm (Y).

For small differences in DNA to be detected between X and Y, the sperm must pass single file through the laser beam, which measures the DNA content of individual sperm.

In orthogonal flow cytometry, a suspension of single cells stained with a fluorochrome is made to flow in a narrow stream intersecting an excitation source (laser beam). As single cells pass through the beam, optical detectors collect the emitted light, convert the light to electrical signals, and the electrical signals are analyzed by a multichannel analyzer. The data are displayed as multi- or single-parameter histograms, using number of cells and fluorescence per cell as the coordinates.

In order to use an orthogonal flow cytrometric system to differentiate between X- and Y-bearing sperm DNA, a beveled sample injection tip and a second fluorescence detector in the forward position is required [Johnson and Pinkel, supra]. This paper is herein incorporated by reference. The modified system allows one to control the orientation of the flat ovoid sperm head as it passes the laser beam. Elimination of the unoriented sperm by electronic gating enhances precision. Typically, 80% of sperm nuclei (without tails) are properly oriented as they pass the laser beam.

In the modified Epics V flow cytometer/cell sorter, hydrodynamic forces exerted on the flat, ovoid mammalian sperm nuclei orient the nuclei in the plane of the sample stream as they exit the beveled injection tip. Fluorescent signals are collected simultaneously by 90 and 0 degree optical detectors, from the edge and flat side of the sperm nucleus, respectively. For sorting, the sample stream is broken into uniform droplets by an ultrasonic transducer. Droplets containing single sperm of the appropriate fluorescence intensity are given a charge and electrostatically deflected into collection vessels. The collected sperm nuclei then can be used for microinjection into eggs. Since the sperm nuclei have no tails, they cannot be used for normal insemination.

Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently, fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head [Pinkel et al. (2), supra]. At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisly on 60 to 80% of the sperm passing in front of the laser beam. The modified Epics V system used in this study can measure the DNA content of tailless sperm from most species at the rate of 50 to 150 sperm per second [Johnson and Pinkel, supra].

Intact sperm (with tails), whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei [Johnson (2), supra]. However, a 90-degree detector can be used to select the population of properly oriented intact sperm to be measured by the 0 degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15 to 20% of intact sperm pass through the laser beam with proper orientation. In this invention, the overall flow rate was approximately 2500 intact sperm per second. The intact X- and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80–90 sperm of each type per second.

It is, of course, of critical importance to maintain high viability of the intact sperm during the sorting process and during storage after sorting but prior to insemination.

Of the factors involved in maintaining sperm viability, the method of staining, the sheath fluid, and the collecting fluid have been found to be especially important.

A nontoxic DNA stain must be selected. A preferred stain is Hoechst bisbenzimide H 33342 fluorochrome (Calbiochem-Behring Co., La Jolla, Calif.). To our knowledge, this fluorochrome is the only DNA binding dye that is nontoxic to sperm. Concentration of the fluorochrome must be minimal to avoid toxicity, and yet be sufficient to stain sperm uniformly and to detect the small differences in the DNA of X and Y sperm with minimal variation. A suitable concentration was found to be 5 μg/ml, but this may be varied from 4 to 5 μg/ml.

The sperm must be incubated with stain at sufficient temperature and time for staining to take place, but under mild enough conditions to preserve viability. Incubation for 1 hr at 35° C. was found to be acceptable, but ranges of 30° to 39° C. would also be effective. Incubation time has to be adjusted according to temperature; that is, 1.5 hr for 30° C.; 1 hr for 39° C.

Sheath fluid used in sorting cells must be electrically conductive and isotonic. A concentration of 10 mM phosphate buffered saline provided the necessary electrical properties, and 0.1% bovine serum albumin was added to enhance sperm viability by providing protein support for metabolism and viscosity for the sperm. The sheath fluid must be free of sugars and excess salts.

Dilution of sperm as occurs in sorting tends to reduce viability of the cells. To overcome this problem, sperm were collected in test egg yolk extender [Graham et al., J. Dairy Sci. 55: 372 (1972)] modified by adjusting the pH and adding a surfactant. Details of the composition of the extender are shown in Example 1. The surfactant is believed to enhance capacitation of the sperm prior to fertilization.

To confirm the DNA content and predict the sex of the offspring of surgically inseminated X or Y sorted sperm fractions, an aliquot of the sorted sperm was sonicated to remove the tails, stained, and the nuclei was reanalyzed for DNA content to predict the proportion of X and Y sperm.

Although the detailed description which follows uses the sorting of rabbit sperm as an example of this invention, it is expected that the sperm of most mammals could be effectively sorted by following these proce-

dures. Those skilled in the art will recognize that minor modifications may be made in the procedure without departing from the spirit and scope of the invention.

Rabbit semen was collected, diluted, and stained with a fluorochrome dye. Sperm were sorted in a modified Epics V flow cytometer/cell sorter.

After being sorted, sperm were surgically inseminated into the uteri of rabbits.

The results obtained by surgical insemination of does with sorted intact sperm are presented in Table I. Recovery of ova 40 hr post-insemination indicated that stained sorted sperm, as well as unstained unsorted sperm, were capable of fertilizing rabbit ova in vivo.

Inseminations were also made to determine the comparability of predicted sex of offspring to phenotypic sex. As the data in Table II indicate, the predictability of the phenotypic sex based on DNA analysis of the separated intact sperm was very high. Reanalysis of the sorted Y population used for insemination indicated that 81% of the sperm were Y-bearing. The sex ratio of offspring from these inseminations was identical to that predicted. These values were significantly different from theoretical 50:50 sex rates ($P<0.003$). Reanalysis of the sorted X-bearing sperm population used for insemination indicated that 86% were X-bearing and 14% were Y-bearing sperm. The phenotypic sex of the offspring from these inseminations was 94% female, which was different from the theoretical 50:50 ($P<0.0003$).

Inseminations were made with sorted X and Y populations that were recombined (recombined X and Y group) immediately before insemination. The assumption was made that the proportions of X and Y in the recombined samples were equal (50:50). The phenotypic sex resulting from the inseminations was 57% female and 43% male (Table II) and was not significantly different from the theoretical (50:50) sex ratio ($P=0.40$).

TABLE I

Fertilizing Capacity of Flow-Sorted Rabbit Spermatozoa After Intrauterine Insemination of Does

Treatment of Sperm	Number of Does Inseminated	Ovulation Points	Eggs Recovered	Eggs Fertilized
Unsorted	2	16	9	9
Sorted	6*	59	46	39

*One doe accounted for 7 recovered and 7 unfertilized eggs

TABLE II

Predicted and Actual Sex Ratios of Offspring After Intrauterine Insemination of Sorted X and Y Chromosome-Bearing Rabbit Sperm

Treatment of Sperm	Number of Does Inseminated	Kindling	Total No. of Young Born	Percentage and Numbers of Offspring: Predicted % Males	Predicted % Females	Actual % Males (N)	Actual % Females (N)
Sorted Y	16	5	21	81	19	81 (17)	19 (4)
Sorted X	14	3	16	14	86	6 (1)	94 (15)
Recombined X and Y	17	5	14	50	50	43 (6)	57 (8)
Total	47	13	51	—	—	47 (24)	53 (27)

The phenotypic sex ratio of offspring born of does inseminated with either sorted X-bearing or sorted Y-bearing sperm was different ($P<0.0002$ for X and $P<0.001$ for Y) from the theoretical (50:50) sex ratio expected from untreated semen.

Embryonic mortality was significant in the does inseminated with sorted intact sperm. With a reasonably high fertilization rate (Table I), one would expect a kindling rate of near 80% and litter size of about six from does of this age and breed. However, the kindling rate across the three treatment groups averaged 28%, with an average litter size of 3.9. The cause of the apparent high rate of embryonic death is though to be due to the fluorochrome binding to the DNA and/or to the effect of the laser beam exciting the DNA bound fluorochrome. Earlier work has shown that sorted vole sperm nuclei that were microinjected into hamster eggs exhibited chromosome breakage in the developing sperm pronucleus [Libbus et al., Mut. Res. 182: 265 (1987)]. Those sperm had been sonicated, stained, sorted, and microinjected, a somewhat more rigorous treatment than the staining and sorting used in this study.

I have demonstrated that DNA can be used as a differentiating marker between X- and Y-bearing sperm, that DNA can be used to accurately predict the sex of offspring from separated X- and Y-bearing sperm populations, and that flow sorting is an effective means for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

EXAMPLE 1

Semen was collected from mixed breed mature bucks by use of an artificial vagina. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer, pH 6.9, to a concentration of 10×10^6 per ml. Bisbenzimide H 33342 fluorochrome was added at a concentration of 5 μg/ml. The samples were incubated for 1 hr at 35° C. Intact sperm were sorted on a modified EPICS V flow cytometer/cell sorter. The stained intact sperm were excited in the ultraviolet (UV; 361 and 364 nm) lines of a 5-watt 90-5 Innova Argon-ion laser operating at 200 mW. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into a test egg yolk extender.

The composition of the extender was N-tris(hydroxymethyl)-methyl-2-amino ethane sulfonic acid, 2.16 g; tris hydroxymethyl aminomethane, 0.51 g; dextrose, 0.1 g; streptomycin sulfate, 0.13 g; penicillin G, 0.08 g; egg yolk, 12.5 ml; Equex STM (Nova Chemical Sales, Scituato, Mass.), 0.5%; and distilled water, 50 ml. This mixture was centrifuged, and only the supernatant was used. The sorted sperm were concentrated by incubating at room temperature for 1 hr, after which the more dilute fraction was removed and the remainder was used for insemination 1 to 4 hr later.

EXAMPLE 2

Mature New Zealand White does were injected with 150 international units of human chorionic gonadotropin (HCG) to induce ovulation, which was expected to occur 10 hr later. Seven hours after treatment with HCG, the does were surgically prepared by injection with Ketamine hydrochloride containing acepromazine and anesthetized under halothane and oxygen. The uterus was exposed by midline incision, and 100 µl of sorted or unsorted sperm was placed into the lumen of the anterior tip of each uterine horn through a 21-gauge needle. Standard management practices were used in caring for the rabbits. These does were sacrificed 40 hr post-insemination; uteri were flushed and recovered eggs evaluated. All fertilized eggs recovered were classified as morula. The results of these experiments are shown in Table I.

EXAMPLE 3

Table II shows the results of inseminations made into the tip of the uterine horn: the number of does that kindled and the phenotypic sex of the offspring compared to the predicted sex. Predicted sex of offspring was based on reanalysis of sorted intact sperm to determine relative DNA content. For reanalysis, the sorted sperm was sonicated for 10 sec and centrifuged at 15,000 g, the supernatant was discarded, and the pellet was resuspended in 9 µM bisbenzimide H 33342. Phenotypic sex of the offspring was determined soon after birth and confirmed at later ages up to 10 weeks. Recombined X and Y is the sorted X and Y sperm populations recombined immediately before insemination.

EXAMPLE 4

Using the methods of Examples 1, 2, and 3, viable swine sperm was sorted into viable X and Y chromosome-bearing populations. Two litters (18 pigs) from surgically inseminated boar semen produced 88% females from X-sorted sperm and 67% males from Y-sorted sperm.

It is understood that the foregoing detailed description is given mainly by way of illustration and that modification and variation may be made therein without departure from the spirit and scope of the invention.

I claim:

1. A method for sorting intact, viable, mammalian sperm into X- and Y-chromosome-bearing populations based on DNA content, the method comprising:

a) staining intact, viable sperm collected from a male mammal with a fluorescent dye capable of selectively staining DNA in living cells by incubating the sperm with the dye at a temperature in the range of about 30°-39° C. for a period of time sufficiently long for staining to take place uniformly but sufficiently short to preserve viability of the sperm;

b) passing the sperm into an electrically conductive and isotonic viability-supporting sheath fluid to form a suspension of sperm which are caused to flow singly in a stream of sheath fluid;

c) passing the sheath fluid containing the sperm before an excitation light source causing the stained DNA to fluoresce;

d) passing the sheath fluid containing the sperm through both a means for detecting the fluorescence of the stained DNA and also a cell sorting means, the means for detecting fluorescence having at least two detectors arranged such that a first detector determines the orientation of sperm on the basis of magnitude of fluorescence and controls a second detector to measure the DNA content of sperm on the basis of magnitude of fluorescence of those sperm that have been determined to be in a preselected orientation;

e) selecting by said cell sorting means the sperm having a DNA content corresponding to a desired chromosome which will produce a desired gender of offspring, and separating the selected sperm from nonselected sperm; and

f) collecting the selected sperm in a viability-supporting collecting fluid.

2. The method of claim **1**, wherein said mammal is a rabbit.

3. The method of claim **1**, wherein said mammal is a swine.

4. The method of claim **1**, wherein said mammal is a bovine.

5. The method of claim **1**, wherein said dye is bisbenzimide H33342 fluorochrome.

6. The method of claim **1**, wherein said incubation is at a temperature of about 39° C. for a period of about 1 hr.

7. The method of claim **1**, wherein said incubation is at a temperature of about 35° C. for a period of about 1 hr.

8. The method of claim **1**, wherein said incubation is at a temperature of about 30° C. for about 1.5 hr.

9. The method of claim **1**, wherein said sheath fluid is phosphate-buffered saline solution, the solution also containing 0.1% bovine serum albumin to enhance sperm viability.

10. The method of claim **1**, wherein said collecting fluid is modified test egg yolk extender.

11. The method of claim **1**, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid prior to being passed before said light source.

12. The method of claim **1**, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid by passing the fluid in a narrow stream through and out of a bevelled injection tip prior to being passed before said light source.

13. A method to preselect the sex of mammalian offspring comprising:

a) sorting sperm according to the method of claim **1**; and

b) inseminating a female mammal of the same species as the male mammal with the selected sperm in the collecting fluid.

14. A method to preselect the sex of mammalian offspring comprising:

a) sorting sperm according to the method of claim **1**; and

b) fertilizing an egg obtained from a female mammal of the same species as the male mammal with the selected sperm in the collecting fluid.

15. The method of claim **1**, further comprising eliminating sperm which are not properly oriented with an electronic gating system before sorting by said cell sorting means.

16. The method of claim **1**, wherein the flow of sperm through the cell sorting means is regulated by an ultrasonic transducer.

17. The method of claim **1**, wherein said sperm are sorted on the basis of X- or Y-chromosome DNA content with about 90% efficiency.

18. The method of claim 1, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid and sperm which are not properly oriented are eliminated by an electronic gating system prior to being passed before said light source.

19. A method to preselect the sex of mammalian offspring comprising:

a) staining intact, viable sperm collected from a male mammal with a fluorescent dye capable of selectively staining DNA in living cells by incubating sperm with the dye at a temperature in the range of about 30°-39° C. for a period of time sufficiently long for staining to take place uniformly but sufficiently short to preserve viability of the sperm;

b) passing the sperm into an electrically conductive and isotonic viability-supporting sheath fluid to form a suspension of sperm which are caused to flow singly in a stream of sheath fluid;

c) passing the sheath fluid containing the sperm before an excitation light source causing the stained DNA to fluoresce;

d) passing the sheath fluid containing the sperm through both a means for detecting the fluorescence of the stained DNA and also a cell sorting means to measure the DNA content of the sperm on the basis of magnitude of fluorescence of the sperm;

e) selecting by said cell sorting means the sperm having a DNA content corresponding to a desired chromosome which will produce the desired gender of offspring, and separating the selected sperm from nonselected sperm; and

f) collecting the selected sperm in a viability-supporting collecting fluid.

20. A method for preparing intact, viable, mammalian sperm for sorting into X- and Y-chromosome-bearing populations based on DNA content, the method comprising staining intact, viable sperm collected from a male mammal with a fluorescent dye capable of selectively staining DNA in living cells by incubating the sperm with the dye at a temperature in the range of about 30°-39° C. for a period of time sufficiently long for staining to take place uniformly but sufficiently short to preserve viability of the sperm.

21. The method of claim 20, wherein said mammal is a swine.

22. The method of claim 20, wherein said mammal is a bovine.

23. The method of claim 20, wherein said dye is bisbenzimide H33342 fluorochrome.

24. The method of claim 20, wherein said incubation is at a temperature of about 39° C. for a period of about 1 hr.

25. The method of claim 20, wherein said incubation is at a temperature of about 35° C. for a period of about 1 hr.

26. The method of claim 21, wherein said incubation is at a temperature of about 30° C. for about 1.5 hr.

* * * * *

X. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by the Board for the proceedings identified in section II of this Appeal Brief. A Related Proceedings Appendix is attached immediately following, starting on the next page, and indicating "(NONE)".

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application Number:	09/744,675
Applicants:	Edward L. Squires, Patrick M. McCue, George E. Seidel
Filed:	January 29, 2001
Title:	Equine System for Non-Surgical Artificial Insemination
TC/A.U:	1634
Examiner:	Carla J. Myers
Assignee:	XY, Inc.
Attorney Docket:	XY-Equine-USNP
Customer No.:	33549
Confirmation No.:	3456

RELATED PROCEEDINGS APPENDIX

(NONE)

XI. CONCLUSION

For the reasons discussed in this Appeal Brief, it is submitted that the Office's rejections of claims 138-145 are erroneous and reversal of the Office is respectfully requested.

Dated this 30th day of June, 2009.

Respectfully Submitted:
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